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SYNTHETIC AND IMMUNOCHEMICAL STUDIES RELATED TO THE
HUMAN H (TYPE 1 AND TYPE 2) AND LEWIS-A
ANTIGENIC DETERMINANTS

by



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A THESIS

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ABSTRACT

The trisaccharides β DGlc(1 \rightarrow 3)[α LFuc(1 \rightarrow 4)] β DGlcNAcOR, (the 4'-epimer of the Lewis-a determinant), α LFuc(1 \rightarrow 2) β DGlc(1 \rightarrow 3) β DGlcNAcOR [the 4'-epimer of the H - Type 1 (Lewis-d) determinant], α LFuc(1 \rightarrow 2) β DGal(1 \rightarrow 4) β DGlcNAcOR (the H - Type 2 determinant) and α LFuc(1 \rightarrow 2) β DGlc(1 \rightarrow 4) β DGlcNAcOR (the 4'-epimer of the H - Type 2 determinant) were synthesized where R is the 8 methoxycarbonyloctyl group, a convenient linking arm for the preparation of artificial antigens and immunoabsorbents. The solution conformations of these compounds were established on the basis of their nuclear magnetic resonance parameters including ^1H and ^{13}C chemical shifts and nuclear Overhauser enhancements. The natural determinants and their 4'-epimers were shown to reside in essentially identical conformations.

Rabbits were immunized with the artificial antigens prepared from the Lewis-a, H - Type 1 (Lewis-d) and H - Type 2 haptens and, also, from their 4'-epimeric analogues. The antisera produced as a result of these immunizations were characterized primarily by the quantitative batch-immunoabsorption assay, a technique which involves the fractionation of antibodies on immunoabsorbents possessing carbohydrate structures related to that of the immunizing

antigen. The result of these investigations require the anti-Lewis-a antibodies to be directed well away from the 4' position of the antigenic determinant. The low levels of hapten-specific antibody produced against the natural H- determinants, compared with the excellent responses obtained against their 4'-epimeric analogues, appear to confirm the expectation that rabbits possess the H-structure as a self determinant.

The H activity in saliva is normally established by examining the inhibition of the agglutination of human H red cells, which possess H - Type 2 determinants at their surface, by the lectin Ulex europaeus. The finding that the H - Type 1 (Lewis-d) and Lewis-b (α LFuc(1 \rightarrow 2) β DGal(1 \rightarrow 3)-[α LFuc(1 \rightarrow 4)] β DGlcNAcOR) structures in the N-deacetylated forms strongly inhibit this agglutination raises the possibility that the Lewis-d and Lewis-b antigens are secreted with the determinants in the amine form.

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CHAPTER I

INTRODUCTION

The discovery at the turn of this century by Landsteiner¹ of the ABO system was a turning point in the science of hematology and a matter of particular importance to blood-transfusion science. It was demonstrated that the serum of a person of either A or B group agglutinated the red cells of the other group, and that the sera of O group people agglutinated cells of both A and B people. The cells of a fourth group of people, termed AB, were found to be agglutinated by the serum of both A and B people but the serum of these did not agglutinate either A or B cells. Agglutination in vivo gives rise to serious pathological conditions referred to as blood-transfusion reactions and can involve extensive lysis of red cells. Its avoidance by proper typing in the ABO system was a major step toward the modern situation where some 20 million litres of blood are collected annually in the western developed countries for transfusion and typing purposes.

With Landsteiner's discovery and the general growth since then of the chemical and biological sciences, the science of hematology rapidly grew to the point that over 160 different human red-cell immunological specificities

are now recognized. At least 13 independent and well-defined blood-group systems are known and some 50 specificities are of clinical importance. At least 5 blood-group systems are known to possess oligosaccharide determinants and this list may grow since carbohydrates, as oligosaccharidic structures, offer a wide assortment of conformationally well-defined structures to serve as recognition sites at cell surfaces.

The ABO and Lewis systems are by far the best characterized of the human blood groups. These determinants are inherited according to the Mendelian genetic laws and arise as the result of the person possessing genes, which gives a code for the enzymes, which are necessary to build the oligosaccharide determinants.

Substances possessing these human blood-group activities occur as oligosaccharides in milk and urine, as complex water-soluble glycoproteins in body secretions and tissue fluids and on the surfaces of cells and tissues, and in the form of water-insoluble glycosphingolipids mainly on the surface of red cells and tissues. It has only been in the last two decades that the structures of these molecules have become known in detail. A review of the extensive structure elucidation work that gave way to this knowledge is beyond the scope of this thesis and the reader is referred to the excellent reviews by Watkins.^{2,3}

The total structure of the oligosaccharide portion of these substances varies depending on whether they are present as the free oligosaccharide, in the glycolipid, or in the glycoprotein. However, when involved as antigens in an immune response, antibodies are developed which, at least in part, possess combining sites for the terminal non-reducing ends of these oligosaccharides. Since, for a given blood specificity, the terminal oligosaccharide structures are the same whether the antigen was a glycolipid or glycoprotein, antibodies raised against one of these structures may recognize the other. This terminal common structural unit is termed the antigenic determinant; i.e., the structure responsible for the particular activity detected.

The chemical structures of the antigenic determinants of the A, B and O active substances are presented in Fig. 1. These tri- and tetrasaccharides provide the structures, in terms of their topographies, which are responsible for the blood-group specificities. From inspection of Fig. 1, it can be seen that these antigenic determinants may possess either a Type 1 or Type 2 linkage. The Type 1 structures are characterized by a β DGal(1 \rightarrow 3) β DGlcNAc sequence at the reducing end of the oligosaccharide unit whereas the Type 2 structures possess the β DGal(1 \rightarrow 4) β DGlcNAc sequence.

<u>Specificity</u>	<u>Structure</u>	
	<u>Type 1</u>	<u>Type 2</u>
O(H)	$\begin{array}{c} \beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc} \\ 1,2 \uparrow \\ \alpha\text{LFuc} \end{array}$	$\begin{array}{c} \beta\text{DGal} \xrightarrow{1,4} \beta\text{DGlcNAc} \\ 1,2 \uparrow \\ \alpha\text{LFuc} \end{array}$
A	$\begin{array}{c} \alpha\text{DGalNAc} \\ 1,3 \downarrow \\ \beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc} \\ 1,2 \uparrow \\ \alpha\text{LFuc} \end{array}$	$\begin{array}{c} \alpha\text{DGalNAc} \\ 1,3 \downarrow \\ \beta\text{DGal} \xrightarrow{1,4} \beta\text{DGlcNAc} \\ 1,2 \uparrow \\ \alpha\text{LFuc} \end{array}$
B	$\begin{array}{c} \alpha\text{DGal} \\ 1,3 \downarrow \\ \beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc} \\ 1,2 \uparrow \\ \alpha\text{LFuc} \end{array}$	$\begin{array}{c} \alpha\text{DGal} \\ 1,3 \downarrow \\ \beta\text{DGal} \xrightarrow{1,4} \beta\text{DGlcNAc} \\ 1,2 \uparrow \\ \alpha\text{LFuc} \end{array}$

Fig. 1 Immunological determinants for O(H), A and B antigens. *The H(Type 1) structure corresponds to the Le^d determinant.

It should be noted at this point that the O designation for red cells that showed neither A nor B activity was adopted by Landsteiner to mean zero activity. With the discovery of the Bombay people⁴, it became apparent that in fact the A⁻B⁻ cells and, therefore, normally termed O cells in fact possess a specific antigenic determinant not present on the red cells of the very rare Bombay types. This structure was eventually established⁵ to be that termed H (Type 2) in Fig. 1. This activity could be related to the α LFuc(1 \rightarrow 2) β DGal terminal unit and, consequently, it was natural enough to assume that the structure termed H (Type 1), shown in Fig. 1, should be so designated; i.e., as an alternate H determinant. This opinion was reinforced by the detection of A or B activities derived from either H (Type 1) or H (Type 2) determinants as precursor structures⁶. However, the overall topographies of these latter two structures are not the same⁷ and antibodies that recognize one of these structures will not necessarily recognize the other and this is well established⁸.

Meanwhile, it became established that the H (Type 1) determinant corresponds to the structure responsible for Lewis-d activity⁹ (Fig. 2). It seemed reasonable, at first, to expect that the α LFuc[(1 \rightarrow 2) β DGal] transferase responsible for the synthesis of the H (Type 2) determinant

would be the same as that involved in the biosynthesis of the H (Type 1) determinant but such expectations did not take into consideration the very different environments about the 2'-OH groups of the β DGal units of the precursor Type 1 and Type 2 disaccharides⁷.

One purpose of this research was to better investigate this and related matters by way of making available, through chemical synthesis, the molecular structures required as substrates for appropriate immunological studies. As pointed out by Lemieux et al.⁷ the fact that the H (Type 1) and H (Type 2) determinants can both serve as substrates for the α DGalNAc and α DGal transferases in the biosynthesis of the A and B determinants is not surprising. That is, their conformational studies indicate that the 3'-OH groups of the β DGal residues in these trisaccharides must be in very near the same environments. A further purpose of this investigation was to better delineate this structural similarity.

In order to more efficiently deal with these subjects, the H (Type 1) determinant will be referred to as the Lewis-d (Le^d) determinant throughout this thesis. In view of this discussion, the use of H will be restricted to reference to the H (Type 2) determinant. It may turn out that the structures listed in Figs. 1 and 2 are all, to a certain degree, only portions of the determinants they

<u>Specificity</u>	<u>Structure</u>
Lewis-c (Le^c)	$\beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc}$
Lewis-a (Le^a)	αLFuc $\downarrow 1,4$ $\beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc}$
Lewis-d (Le^d) *	$\beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc}$ $1,2 \uparrow$ αLFuc
Lewis-b (Le^b)	αLFuc $\downarrow 1,4$ $\beta\text{DGal} \xrightarrow{1,3} \beta\text{DGlcNAc}$ $1,2 \uparrow$ αLFuc

Fig. 2 Immunological determinants for the Lewis antigens. *As seen in Fig. 1, the Le^d and H(Type 1) determinants are the same structure.

represent. However, it seems best to reserve such considerations to the time that experimental evidence exists in support of this possible eventuality.

This thesis will also examine certain aspects of the Lewis system of human blood-group specific antigens. These antigens are characteristic of epithelial cells from which they are transported by the plasma to the endothelia and thus also found in low concentration on the red cells. The structures of the Lewis antigenic determinants are presented in Fig. 2. These oligosaccharides all possess the Type 1 linkage.

The establishment of these and other determinants as being either present or absent on red cells, tissues, etc., forms the basis of serology. The intrusion of synthetic carbohydrate chemists into the field of serology in recent years has been more a matter of necessity than one of curiosity. Blood-group typing work has been seriously hindered by the enormous difficulties encountered in obtaining even milligram quantities of blood-group-specific active substances from natural sources. Even in the best of cases, these samples are usually heterogeneous mixtures of closely related and often cross-reacting compounds. The preparation of synthetic blood-group active oligosaccharides not only alleviates this problem but, and more importantly, offers the only solution to what the author

likes to refer to as the "black box" problem which plagues the field of serology and related areas. This type of problem can best be illustrated by example.

In the field of serology, a person is characterized as, for example, Lewis-a positive, not because he is shown to possess the trisaccharidic antigenic determinant β DGal-(1 \rightarrow 3)[α LFuc(1 \rightarrow 4)] β DGlcNAc on his cells or tissues but because antibodies which occurred in the serum of a patient named Lewis were found to react with his cells or tissues. Indeed, the anti-Lewis-a activity of certain sera was discovered, in 1946, long before the structure of the antigen became known. A Lewis-a antigen is thus defined, clinically, as a substance that reacts with an anti-Lewis-a serum and, for successful typing, there is no need to know the structure of the antigenic determinant that binds with the antibodies. The availability of well characterized and standardized antibodies specific for the Lewis-a antigenic determinant is sufficient for reliable typing.

Anti-Lewis-a antibodies can be obtained from the serum of animals immunized with "Lewis-a-active" substances such as red cells or saliva from individuals who have been designated as Lewis-a positive; i.e., people who possess on their cells, or in their saliva, the antigens responsible for the reaction with the anti-Lewis-a antibodies. Such sources contain, however, not only the Lewis-a

antigen but also a wide variety of other antigens such that the animal will synthesize not only the desired antibodies, but a myriad of others directed towards these accompanying antigens. With luck, a source of Lewis-a-active glycoprotein may be obtained from a natural source such as ovarian cysts. Even when highly purified, however, such glycoproteins are still heterogeneous structures which will elicit the production of correspondingly heterogeneously-specific antibodies, directed against both the protein and other antigenic determinants that may be present on its surface. The anti-serum derived from animals immunized with these "ideal-substances" will thus contain antibodies that react with unknown determinants that are of other than Lewis-a specificity.

If the red cells of a person possess one of these other antigenic determinants but not the Lewis-a trisaccharide, he could appear to be Lewis-a positive by this anti-serum even though he is, in fact, Lewis-a negative. To purify such an anti-serum to the point of mono-specificity, one should ideally possess a substance that presents uniquely the Lewis-a determinant. But such substances, as discussed above, are generally not available from nature. Therefore, the practice has been to adsorb the serum with a range of appropriately

chosen Lewis-a negative red cells to the point where interfering cross-reactive antibodies were effectively removed. This procedure, however, still leaves in the serum antibodies of unknown specificities whose antigens were not present on the panel of cells chosen for the adsorptions. This situation can be termed a "black box" problem since it is one of trying to identify an unknown antigen (a black box) with an antiserum whose specificities are themselves not fully characterized (another black box).

An approach to the solution of these basic problems, as regards oligosaccharidic determinants, has been developed in these laboratories.^{10,11} Once the structure of the antigenic determinant responsible for a certain blood-group specificity has been determined (or postulated), this determinant is then prepared, as its 8-methoxycarbon-yl-octyl glycoside, through a multi-step synthesis. This oligosaccharidic glycoside is then a hapten; i.e., a low molecular weight compound that lacks antigenicity but is capable of reacting specifically with the appropriate antibody. The choice of this long-chain aliphatic aglycon was based on the need of such a structure to serve as a bridging arm for the preparation of immunoadsorbents and artificial antigens. The preparation of artificial antigens by attachment of the hapten to a suitable carrier

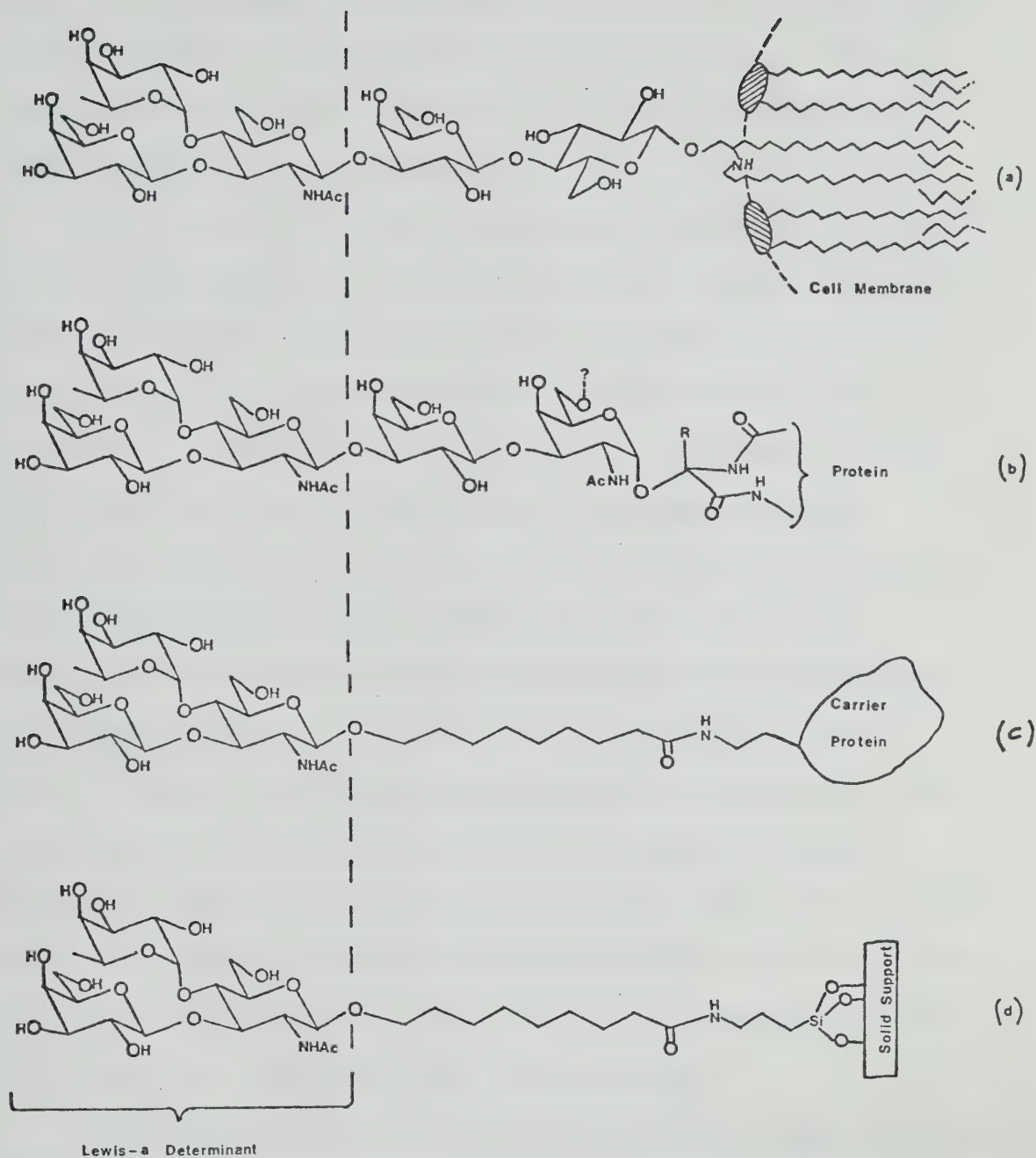


Fig. 3 Illustration of natural and synthetic Le^a active substances: a, a glycosphingolipid hydrophobically bound to a cell-membrane lipid bilayer; b, a glycoprotein; c, an artificial antigen; d, an immunoabsorbent.

molecule allows the immunization of animals to raise antibodies specific to the carbohydrate determinant. The immunoadsorbent then allows the isolation of these antibodies from the animal serum.

The potential of such an approach may be appreciated by referring to Fig. 3 where the structures for the natural Lewis-a antigens, present as glycolipid at the surface of a red cell (a) or glycoprotein in secretions (b), are illustrated. The artificial antigen (c) is simply a chemist's approximation of the natural antigen. If the synthetic oligosaccharide is indeed identical to the antigenic determinant in the natural antigen, it can be appreciated that the antibodies raised against the synthetic (mono-specific) antigen, and isolated by virtue of their affinity for the corresponding immunoadsorbent (d), should bind the antigenic determinants of the natural antigens. These antibodies are then specific for an exact and known chemical structure and thus allow the serologist to positively establish the presence, or absence, of this structure on a specimen under investigation.

Aside from this and many other practical applications, the ability to prepare an essentially unlimited variety of synthetic haptens, antigens and immunoadsorbents allows for a more fundamental investigation into the nature of antibody-antigen interactions.

The research that will be reported in this thesis was initiated, essentially, as a result of the observation¹² that antibodies raised, as just described, against the Lewis-b (Le^b) artificial antigen, and hence supposedly specific for the Le^b tetrasaccharide, cross-reacted to a high degree with both the Lewis-a (Le^a) and Lewis-d (Le^d) trisaccharides. This situation may be rationalized by a consideration of the structures of these determinants (Fig. 2) in that both the Le^a and Le^d trisaccharides are constituents of the Le^b tetrasaccharide. These anti- Le^b antibodies could, in addition, be separated into three classes: those reacting with both the Le^a and Le^b determinants, both the Le^d and Le^b determinants, and only the Le^b determinant.

It was thus clear that such "mono-specific" antibodies are a micro-heterogeneous mixture of different molecules each of which, while "specific" for the structure of the antigenic determinant to which it was raised, recognizes it in a different manner. This situation may be likened to that of an object being photographed from many different angles. Each picture, while different in appearance, still identifies that object. In the same manner, each separate antibody that reacts with a determinant defines a separate specificity for that determinant.

One goal of the research reported in this thesis was to clarify the extent to which such hapten-specific antibodies might cross-react with similar structures. Such cross-reactivity might provide the basis for new methods for the fractionation and characterization of heterogeneous antibody populations. In addition, information regarding the immunodominant parts of the determinants themselves might be gained which would allow some limits to be placed on the spectrum of potentially cross-reactive structures.

The specificities of the antigenic determinants of the Le^a , Le^d and H human blood-groups, and their corresponding antibodies, have been examined using this approach. Analogues of these trisaccharides possessing a D-glucose, instead of the natural D-galactose, residue were selected as synthetic objectives as it had long been appreciated that anti- β -D-galactopyranosyl antibodies bind a β -D-glucopyranosyl unit extremely weakly if at all.¹³ Should the binding of the blood-group specific antibodies with the natural antigens involve any substantial binding of the β -D-Gal unit, the gluco-analogues of the determinants would therefore be expected to be very poor inhibitors.

The stereochemical representations of the structures under consideration are shown in Fig. 4. Evidence that these trisaccharides assume, in solution, the conformations as shown will be presented later. Comparison of the

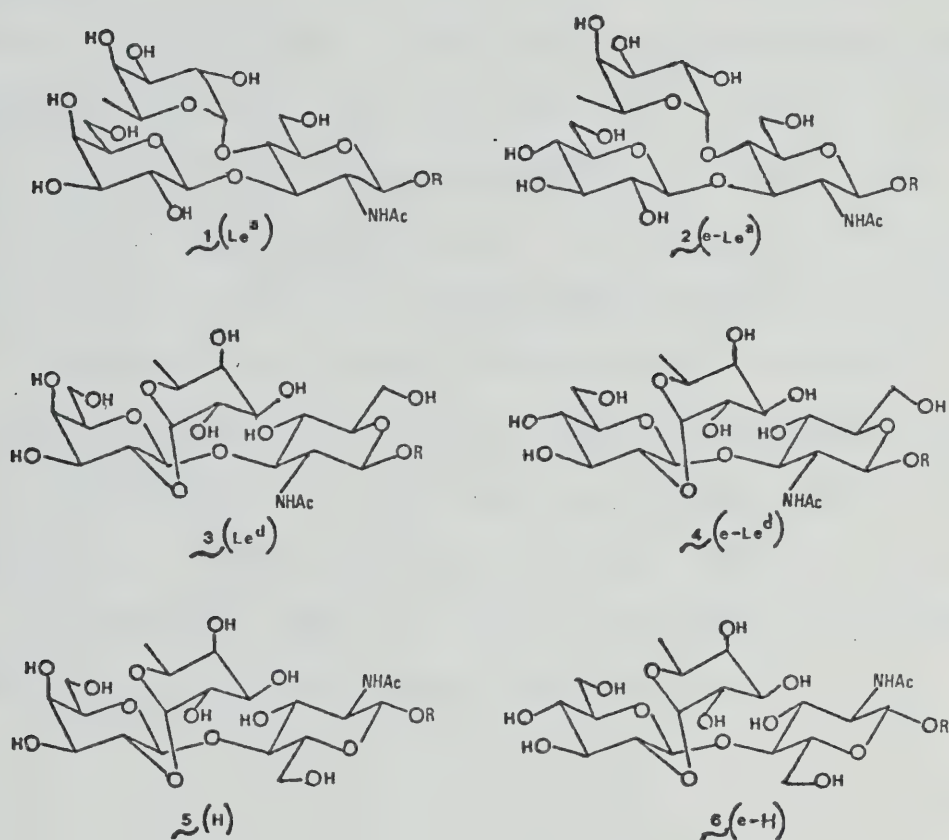


Fig. 4 Structures and approximate conformations of the Le^a , Le^d and H haptens, and their gluco-analogues: 4'-epi-Lewis-a (e-Le^a), 4'-epi-Lewis-d (e-Le^d) and 4'-epi-H (e-H).

structures of the "natural" haptens (1, 3 and 5) with those of their required gluco-analogues (2, 4 and 6) shows each pair to be epimeric at the 4' position. For brevity, compounds 2, 4 and 6 will be referred to here as 4'-epi-Lewis-a, 4'-epi-Lewis-d and 4'-epi-H, respectively; or simply e-Le^a, e-Le^d and e-H. The Le^a 10 and Le^d 14 haptens, antigens and immunoabsorbents had already been prepared in these laboratories. The syntheses of the e-Le^a, e-Le^d, H and e-H haptens would then be required.

Aside from the primary objective of providing haptens for the immunochemical studies, this synthetic work has helped to provide some new insights into the mechanisms of glycosylation reactions. Some new synthetic methodologies have also been introduced which should prove useful to other workers in this field.

CHAPTER II

OLIGOSACCHARIDE SYNTHESIS

A. General Synthetic Strategies

The main difficulty in the synthesis of complex oligosaccharides is to achieve the establishment of the required glycosidic linkages in good yield. The problems of blocking and deblocking attendant to this work with multifunctional substrates is not trivial. Considerable planning is required in terms of choice of blocking groups with regard to both a maintenance of the hydroxyl group to be glycosylated in as reactive a form as possible and the keeping of access to the positions to be glycosylated in the order necessary for overall success.

Although a vast compendium of methods exists for the stereospecific glycosylation of simple alcohols, few of these have survived the transition to syntheses at the oligosaccharide level. A synopsis of these "best methods" which have proved to be useful in the syntheses of oligosaccharides related to the A, B, O(H) and Lewis blood groups in the past will be presented in this section.

Much of the author's insight into the mechanisms of these glycosylation reactions was obtained as a result of experiences encountered during the present research. A

discussion of the mechanisms of these reactions will, however, be offered at this point. It is hoped that, in this manner of presentation, the reader will acquire a clearer understanding of both the synthetic strategies that were used and the seemingly anomalous products obtained in some of the glycosylation reactions.

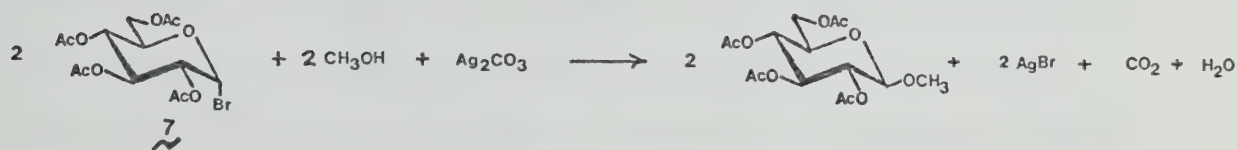
The structures of the target haptens 2, 4, 5 and 6 were presented in Fig. 4. The intersugar glycosidic bonds in these structures may be broadly classified as either 1,2-trans- β or 1,2-cis- α linkages: the cis and trans designations referring to the relative stereochemistry of O-1 and O-2. Different strategies are required for the formation of these two types of linkages.

1. The 1,2-trans- β -glycosidic Linkage

a. The Koenigs-Knorr Reaction^{15,16,17}

The Koenigs-Knorr reaction is by far the most commonly used method for the establishment of the glycosidic linkage. Although originally reported¹⁵ as the reaction between an acetylated glycosyl halide and an alcohol in the presence of either silver carbonate or silver oxide, the Koenigs-Knorr reaction is now described more broadly as the condensation of an O-protected glycosyl halide and an alcohol in the presence of any halophilic heavy-metal ion.

The classical reaction¹⁵ may be illustrated by the condensation of acetobromoglucose and methanol, in the presence of silver carbonate, to provide methyl 2,3,4,6-tetra-O-acetyl- β -D-glucopyranoside (Scheme 1). The reaction, nowadays, is normally carried out in the presence



Scheme 1

of a dessicant (either Drierite or molecular sieve) to remove the water formed by the neutralization of the hydrogen bromide.

The greater thermodynamic stability of pyranose rings bearing electronegative substituents at the 1-position in the axial orientation has been termed the anomeric effect.¹⁸ Under the usual reaction conditions for the preparation of O-protected glycosyl halides, the more

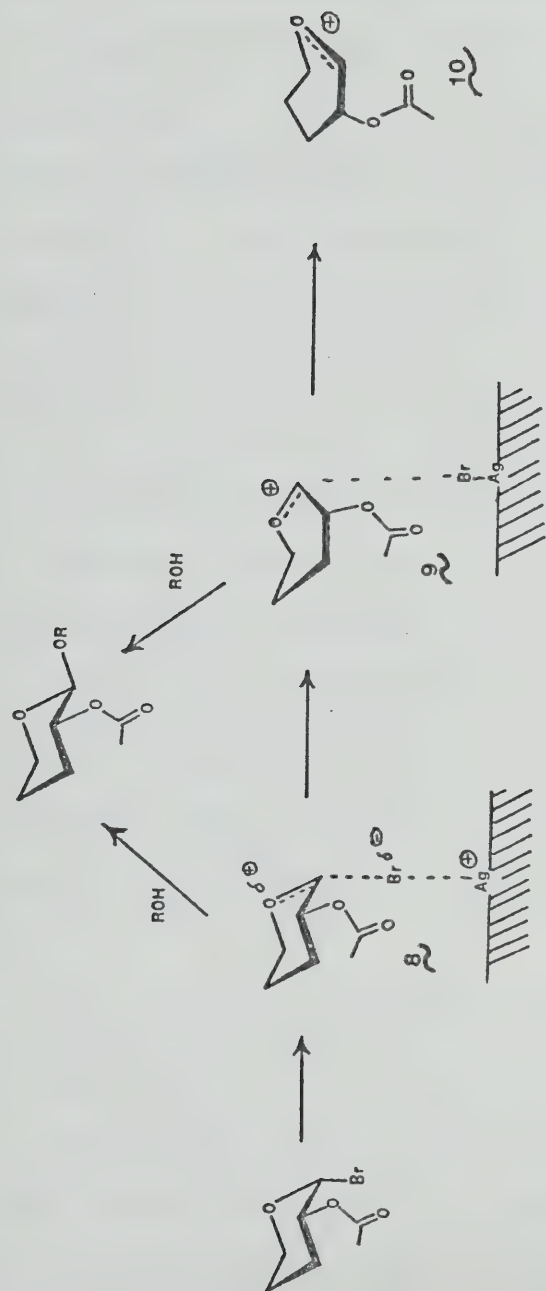
stable α -anomers are obtained. The formation of the β -glycosides in the Koenigs-Knorr reaction from such halides then proceeds with net inversion of configuration at the anomeric center. The yields are generally moderate to good for primary alcohols but are often rather poor with secondary and more hindered alcohols. The reaction, as shown in Scheme 1, has consequently found little use in oligosaccharide synthesis. An examination of the reasons for the failure of this reaction to produce glycosides of sterically-hindered or poorly nucleophilic alcohols will, however, provide the framework for an understanding of those modifications that have proved successful.

Unfortunately, only fragmentary data concerning the mechanism of this reaction have so far become available.^{16,17} Particular difficulties arise in this area owing to non-quantitative yields, the use of heterogeneous reaction conditions and, until recent years, insurmountable problems of quantitative analysis. These strongly limit the use of physical organic methods for studying the reaction. Enough qualitative information, particularly the identity of side-products, has, however, been obtained to allow the postulation of mechanisms.^{16,17}

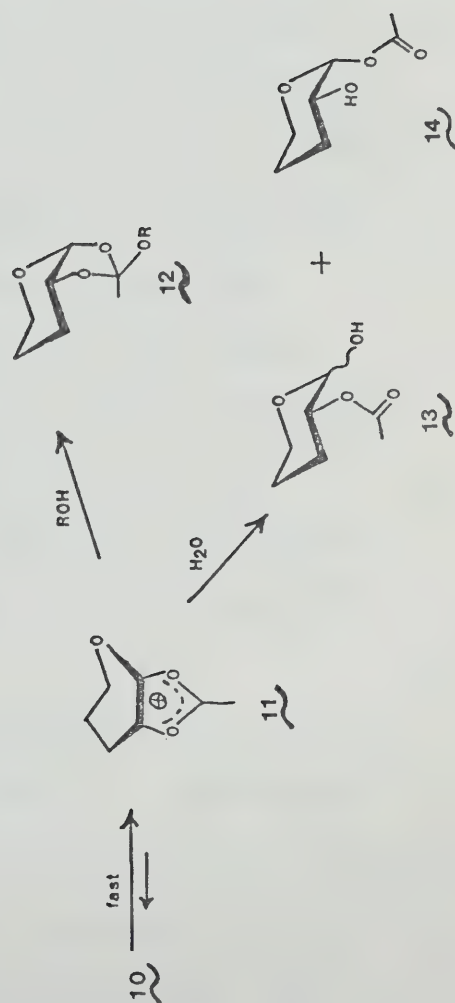
Although the addition of silver salts was originally intended only for the purpose of keeping the reaction conditions neutral, the silver ions are now known¹⁶ to act as

promoters for the reaction. Owing to the heterogeneity of the system, the catalysis occurs on the phase interface and so the course of the reaction will depend on the size of the particles, the manner in which they were prepared, the speed of stirring, etc. (the difficulties of standardization of all these factors sometimes render the results of these reactions difficult to reproduce).

The proposed mechanism¹⁶ (Scheme 2) involves, in the first stage, a silver-ion assisted heterolysis of the C-1 to halogen bond and is consequently discussed in terms of an S_N^1 mechanism. In the few cases where the alcohol has been found to participate in the rate-determining step, this has been attributed to a "push-pull" mechanism (8). That only trans-glycosides are normally obtained in spite of the usual S_N^1 mechanism has been explained¹⁶ in terms of a direct reaction between the alcohol and the intimate ion-pair 9 where the α -side is shielded. β -Glycoside formation is likely to occur only when the alcohol can intercept either 8 or 9. When the alcohol is either present in too low a concentration or not sufficiently nucleophilic to intervene at these stages, the ion pair separates to give the free glycosyl carbonium-ion 10, a matter which is facilitated by the participation of the lone-pair electrons of the ring oxygen in charge stabilization. This carbonium ion then rapidly rearranges to the thermodynamically more



Scheme 2



stable dioxolan-2-ylum ion $\underline{11}$, the trivially-named acetoxonium ion, by participation of the neighbouring 2-acetoxy group. Although many competing reaction pathways, notably involving proton elimination and the scavenging of water, are available to ambident cations such as $\underline{11}$, their kinetic products with alcohols are known^{19,20} to be mainly orthoesters ($\underline{12}$). The reasons for the failure of the silver carbonate (or silver oxide) reaction to produce β -glycosides from complex alcohols is that these are too unreactive to intercept the intermediates $\underline{8}$ or $\underline{9}$ and the reaction is diverted by way of the acetoxonium ion $\underline{11}$. Aside from the products arising from its hydrolysis ($\underline{13}$ and $\underline{14}$) (Drierite picks up water relatively slowly), $\underline{11}$ reacts predominantly to give the orthoester $\underline{12}$. If the neutralization of hydrogen bromide is sufficiently rapid and efficient, the orthoester accumulates as the major product. Indeed, orthoesters are often isolated in good yields.¹⁶

It should be noted that β -glycoside formation in the Koenigs-Knorr reaction, as just described, is independent of neighboring group participation, the participation resulting almost exclusively in orthoester formation. It will not be surprising, then, to find that the modifications of the Koenigs-Knorr reaction that have proved successful in the synthesis of complex oligosaccharides, where the

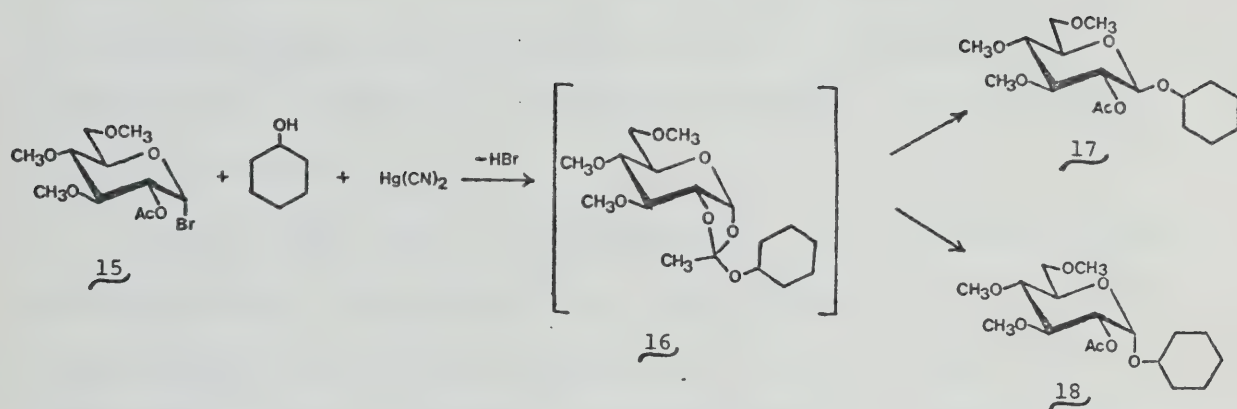
saccharidic alcohols will be too unreactive to intercept intermediates like 8 or 9, are exactly those that maintain the reaction conditions sufficiently acidic so as to render the orthoester 12 unstable and promote its further reaction.

b. The Helferich Reaction²¹

In all but one²² of the published syntheses of ABO(H) and Lewis blood-group oligosaccharides, the establishment of the 1,2-trans- β -glycosidic linkage has involved the condensation of a 2-O-acyl glycosyl halide with an alcohol in the presence of Hg(II) salts. This modification of the Koenigs-Knorr reaction, which employs mercuric cyanide and/or mercuric bromide as promoters, has been termed the Helferich reaction. Overwhelming support that all these condensations proceed via the intermediacy of orthoesters has been obtained by Wallace and Schroeder.^{23,24,25}

These investigators have examined, in detail, the course of the reaction of 2-O-acetyl-3,4,6-tri-O-methyl- α -D-glucopyranosyl bromide (15) with cyclohexanol in the presence of mercuric cyanide as promoter.²⁵ A summary of their key results that are of concern to this discussion are presented in Fig. 5.

The reaction was found²⁵ to exhibit a first-order kinetic dependence on the glycosyl bromide (15) and mercuric cyanide but was independent of the cyclohexanol concentration. Detailed kinetic studies and product analysis



<u>mole ratios</u>			<u>initial products (%)</u>			<u>final glycosidation products (%)</u>	
cyclohexanol	<u>15</u>	Hg(CN) ₂	<u>16</u>	<u>17</u>	<u>18</u>	<u>17</u>	<u>18</u>
30	: 1	: 1	23	73	4	94	6
22.5	: 1	: 1	36	60	4	93	7
15	: 1	: 1	45	49	6	93	7
7.5	: 1	: 1	53	39	8	93	7

Fig. 5 Some results obtained by Wallace and Schroeder²⁵ in their mechanistic investigation of the Helferich reaction.

allowed the orthoester 16 to be identified as an important initial product in the reaction. Even in the presence of a 30-fold molar excess of cyclohexanol, a relatively nucleophilic alcohol, the orthoester 16 accounted for 23% of the initial condensation product (Fig. 5). Reducing the alcohol concentration to a 7.5 molar excess resulted in a progressive increase in orthoester formation to 53% of the initial products.

Even in the case of cyclohexanol at a 7.5 molar excess concentration, the bulk of the β -glycoside isolated in the final product was derived from the orthoester. Wallace and Schroeder²⁵ could isolate the orthoester 16 in good yield when the reaction was performed in the presence of added mercuric oxide as buffer. This orthoester was then shown to rearrange under the Helferich conditions ($\text{Hg}(\text{CN})_2 \cdot \text{HBr}$) to provide mainly the β -glycoside 17. Formation of some 2-O-deacetylated α and β -glycosides, along with an equivalent amount of acetoxycyclohexane, was also observed.

An extrapolation of these observations to the case of a disaccharide synthesis under the Helferich conditions, where a poorly nucleophilic alcohol is present only in near stoichiometric concentration with the halo-sugar, must lead to the conclusion that orthoester formation will be by far the major initial process. The observed products will then be a function of the properties of this orthoester.

The establishment of orthoesters as initial products in these glycosylation reactions leaves open two approaches for the improvement of yields in the synthesis of 1,2-trans- β -glycopyranosides. One approach would be to design a glycosyl halide, with an acyl participating group at the 2-position, whose orthoesters would be unstable relative to the dioxolan-2-ylum ion. The contribution of the orthoester to the overall reaction might then be insignificant. Such an approach using intramolecularly stabilized dioxolan-2-ylum ions, in the form of spiro-acylorthoesters, has been pursued during this research.²⁶ Although this investigation yielded some new and highly labile compounds with interesting conformational properties, an improved glycosylation method was not realized. Since the results of this investigation²⁶ are not pertinent to the immunochemical aspect of this thesis, these are presented separately in the appendix.

The remaining approach would be to learn how to control the acid-catalyzed reactions of sugar 1,2-orthoesters. Kochetkov and co-workers^{27,28} have examined the rearrangement of orthoesters under a wide variety of conditions and found the course of the reactions to be highly sensitive to the nature of the catalyst and the solvent as well as the sugar and the alcohol. The best yields of orthoester \rightarrow β -glycoside transformation were achieved using either

mercuric bromide in nitromethane or lutidinium perchlorate in refluxing chlorobenzene.^{27,28} With secondary saccharidic alcohols, the yields of 2'-O-acetyl- β -disaccharides were usually moderate (20-40%). Significant amounts of 2'-O-acetyl- α -disaccharide as well as de-O-acetylated α and β disaccharides were frequently produced. Their most notable observation²⁸ was that the orthoester $\rightarrow\beta$ -glycoside transformation generally occurs more rapidly, and in better yield, in the presence of added alcohol and thus, strictly speaking, the reaction is likely not a rearrangement but an intermolecular reaction.

A small degree of order has been brought to this chaotic state of affairs by the recent work of Garegg and Kvarnstrom.^{29,30} These investigators examined the influence of the aglyconic alcohol on the course of the orthoester 'rearrangement'. Their findings of concern to this discussion are presented in Fig. 6.

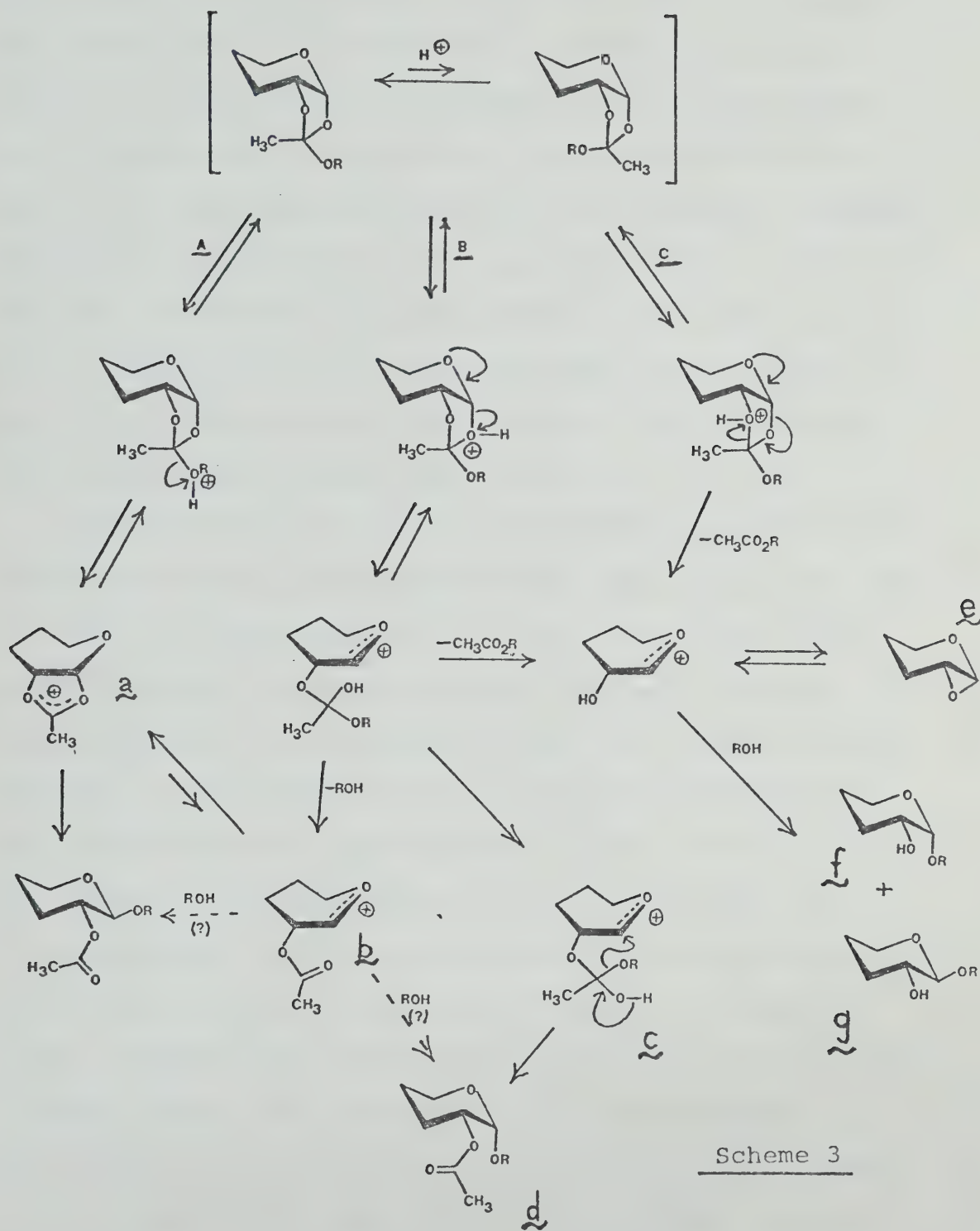
From Fig. 6, the nature of the alcohol can be seen to be critical to the outcome of the reaction. Increasing the number of electronegative substituents on the methyl group of the aglyconic alcohol can be seen to result in the increased formation of the α -glycoside. This has been interpreted²⁹ in terms of a decreased basicity of the alcoholic oxygen atom in the orthoester (see later). Increased steric hinderance of the alcohol also affects the stereo-

R	yield of glycosides (%)	% composition	
		β	α
$-\text{CH}_2\text{CH}_3$	100	100	0
$-\text{CH}_2\text{CH}_2\text{Cl}$	100	84	16
$-\text{CH}_2\text{CHCl}_2$	100	50	50
$-\text{CH}_2\text{CCl}_3$	100	33	67
	62 ^a	100	0
	40 ^b	66	33

Fig. 6 The results obtained by Garegg and Kvarnstrom^{29,30} in various orthoester glycoside transformations. ^aThe remaining products were the 2-O-deacetylated α and β -glycosides. ^bThe remaining products were not identified.

chemical outcome of the glycosylation as well as the yield.³⁰ Considering that saccharidic alcohols are not only highly hindered but also heavily substituted by electronegative oxygen atoms, it is not surprising to find that such glycosylations are rather unpredictable reactions. The complexity of the situation may be further appreciated by referring to Scheme 3 where what the author considers to be the reasonable modes of reaction of sugar 1,2-orthoesters in the presence of an acidic catalyst (for simplicity, denoted by a proton) have been presented. This Scheme has deliberately been further simplified by the omission of counter-ions and solvent. It should be stressed that Scheme 3 encompasses only the 'productive' reactions and not the 'destructive' reactions (proton eliminations, acetoxonium ion migrations, formation of anomeric cyanides, isonitriles, or cyanoalkylidene compounds when $\text{Hg}(\text{CN})_2$ is used) that will become important routes of reaction should all the 'productive' processes become too slow.

With reference to Scheme 3, the production of β -glycosides in either the Helferich or orthoester rearrangement reactions should proceed via the dioxolan-2-ylum ion a. The frequent success of these reactions may be attributed to the relative stability and ease of formation of this species. The production of a, however, requires at least partial protonation of the exocyclic oxygen of the



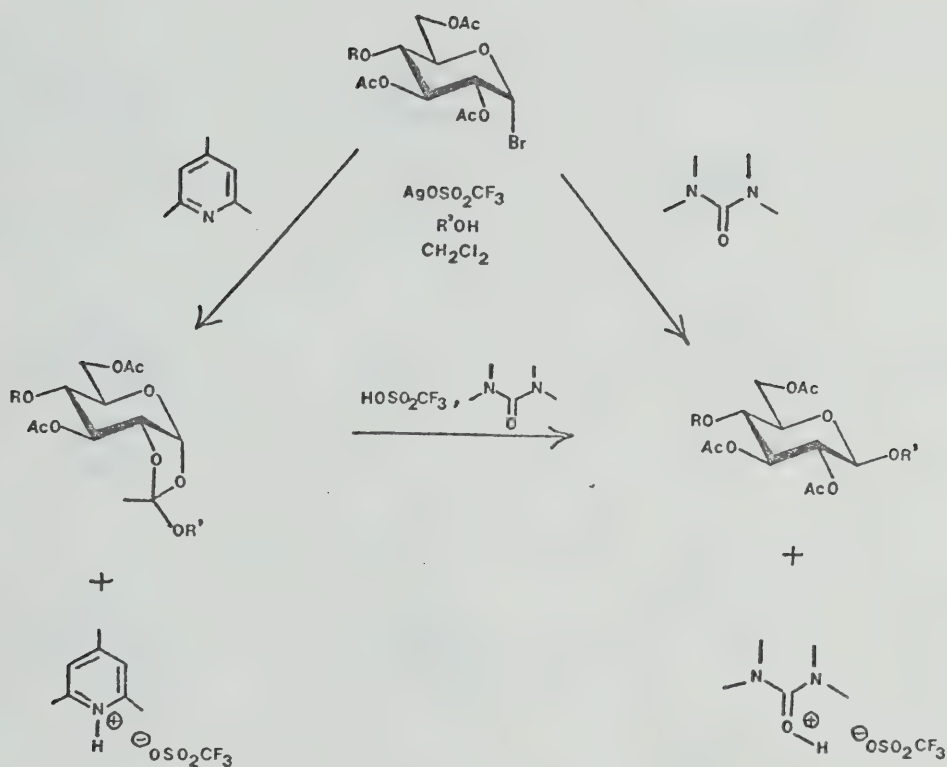
Scheme 3

orthoester, presumably via route A. Decreasing the basicity or accessibility of this oxygen, as shown by Garegg and Kvarnstrom,^{29,30} results in an increase in the production of α -glycosides or deacetylation products which may be interpreted in terms of a competition by routes B and C. Whether 2-O-acyl- α -glycoside (d) formation proceeds through the intermediates b or c is not known. A consideration of the expected relative stabilities of the ions a and b, coupled with the high "effective concentration" of the 2-O-acetyl group in b would lead to the expectation that b would more rapidly rearrange to a.

The production of acetylated alcohols and 2-O-deacetylated products in near equal amounts can be rationalized in terms of either routes B or C and a clear distinction cannot be made. Support for these two routes has come mainly from Lemieux and Morgan³¹ who have investigated the reaction of 3,4,6-tri-O-acetyl-1,2-O-(alkyl orthoacetyl)- α -D-glucopyranose in the presence of strong proton acids and alkanol. Using methylene chloride as solvent and p-toluenesulphonic acid as catalyst, alkyl 3,4,6-tri-O-acetyl α and β -D-glucopyranosides were produced in 70% and 20% yields, respectively. The yield of alkyl acetate was nearly quantitative. The reaction of 1,2 anhydrosugars (e), such as Brigl's anhydride,³² with alcohols in the presence of acid catalysts is also known^{33,34} to provide mixtures of α and β glycosides (f and g).

While it is clear that the intermediacy of orthoesters can explain the diversity of products obtained in glycosylations performed under acidic Koenigs-Knorr conditions, it is perhaps less evident that these reactions do proceed almost entirely via the orthoester. Two recent articles should clear up any remaining doubts and these are summarized in Figs. 7 and 8. Banoub and Bundle³⁵ have found (Fig. 7) that while the reaction of peracetylated disaccharide bromides with silver triflate in the presence of a small excess of 2,4,6-collidine results only in the production of orthoester, the use of tetramethylurea in place of collidine results almost exclusively in β -glycoside formation. Furthermore, treatment of the orthoester obtained in the collidine reaction with triflic acid-tetramethylurea complex resulted in its conversion to the β -glycoside. When the tetramethylurea reaction was conducted at -20°C , the orthoester intermediate was observed by $^1\text{Hnmr}$. These differences in reaction can only be interpreted in terms of the pK_a 's of the conjugate acids of the bases involved in the reaction. Thus, the use of collidine suffers from the same problems as the use of silver carbonate in the original Koenigs-Knorr reaction: the reaction conditions are not sufficiently acidic to promote the orthoester rearrangement.

Garegg and Norberg³⁶ have recently proposed the use of perbenzoylated glycosyl halides as glycosylating agents.



R = peracetylated glycopyranoside

R' = $(\text{CH}_2)_8\text{COOCH}_3$

Fig. 7 Variation of the outcome of a Koenigs-Knorr reaction when either 2,4,6-collidine or tetramethylurea are used as base.³⁵

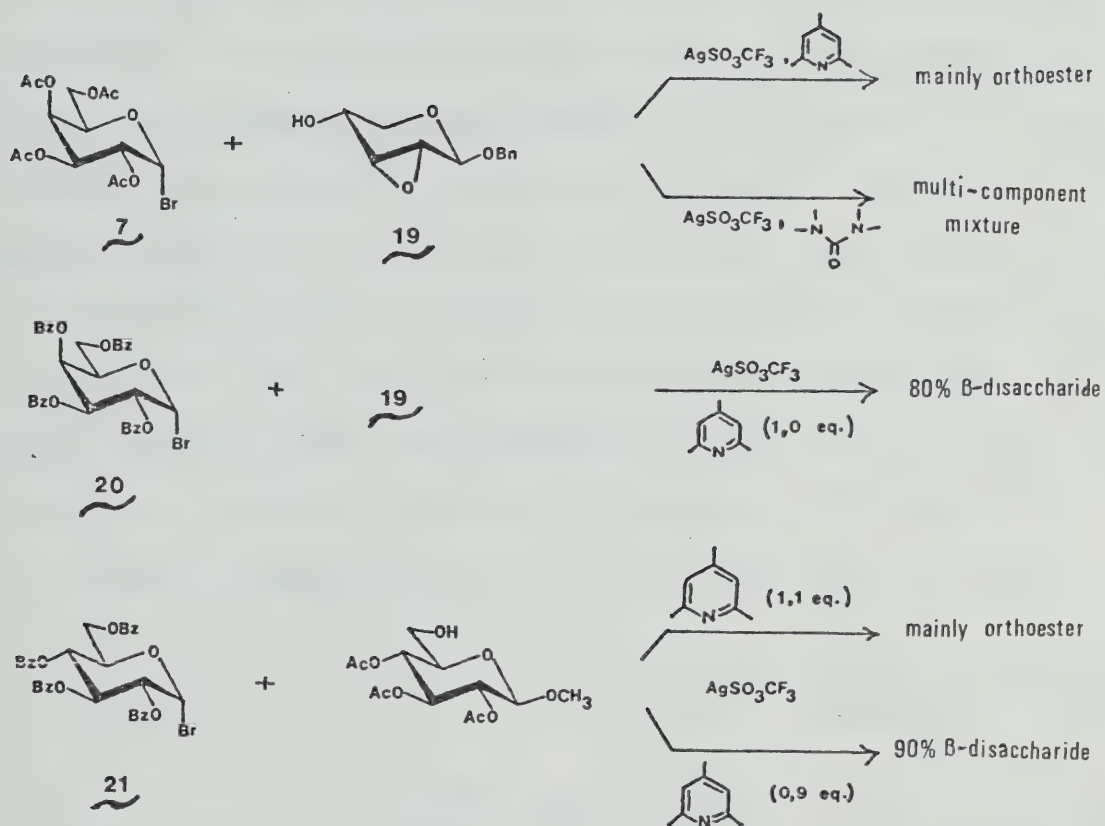
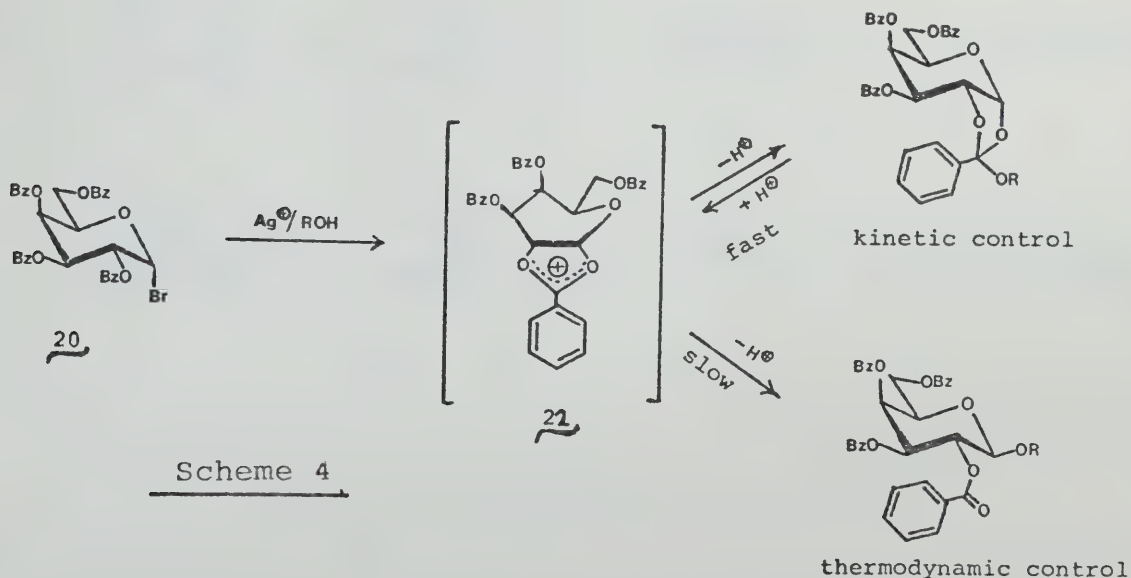


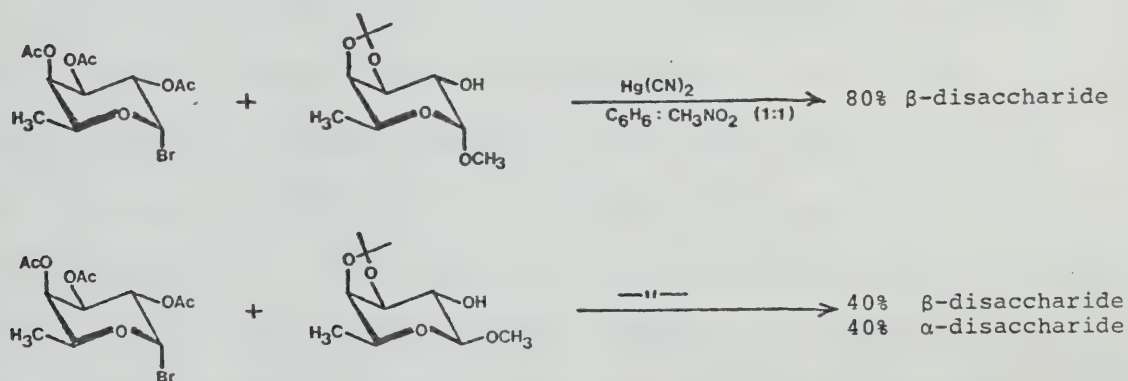
Fig. 8 Some glycosylation reactions reported by Garegg and Norberg.³⁶

After finding (Fig. 8) that the use of the silver triflate-tetramethylurea combination failed to provide any significant glycoside from 7 and 19, and more basic conditions (collidine) yielded only orthoester, they engaged the alcohol 19 in the Koenigs-Knorr reaction with benzobromogalactose (20) and obtained an excellent yield of 1,2-trans- β -glycoside. In the glycosylation with 21, the amount of collidine present proved critical to the outcome of the reaction; the orthoester mainly being formed under basic conditions and the β -glycoside under acidic conditions.

The high yields of β -glycosides obtained in these glycosylations with the per-benzoylated bromide 20 are clearly the result of the diversion of the orthoester rearrangement to route A (Scheme 3) leading to the particularly stable 2-phenyl-dioxolan-2-ylum (benzoxonium) ion



22 (Scheme 4). The extra stability of this species should result in an increase in its rate of formation from the orthoester as compared to that for the 2-methyl-dioxolan-2-ylum (acetoxonium) ion. In further support of this contention, there is the report by Kochetkov²⁷ that in the one case where the rearrangement of an orthoacetate and an orthobenzoate were directly compared (using HgBr_2 as catalyst and 1,2,3,4-tetra-O-acetyl- β -D-glucopyranose as alcohol), the yield of β -linked disaccharide was almost twice as high with the benzoate (93%) as with the acetate (54%). The implicit suggestion from the report of Garegg and Norberg³⁶ is that, at least in some cases, even collidinium triflate is sufficiently acidic to effect the orthobenzoate \rightarrow β -glycoside transformation.



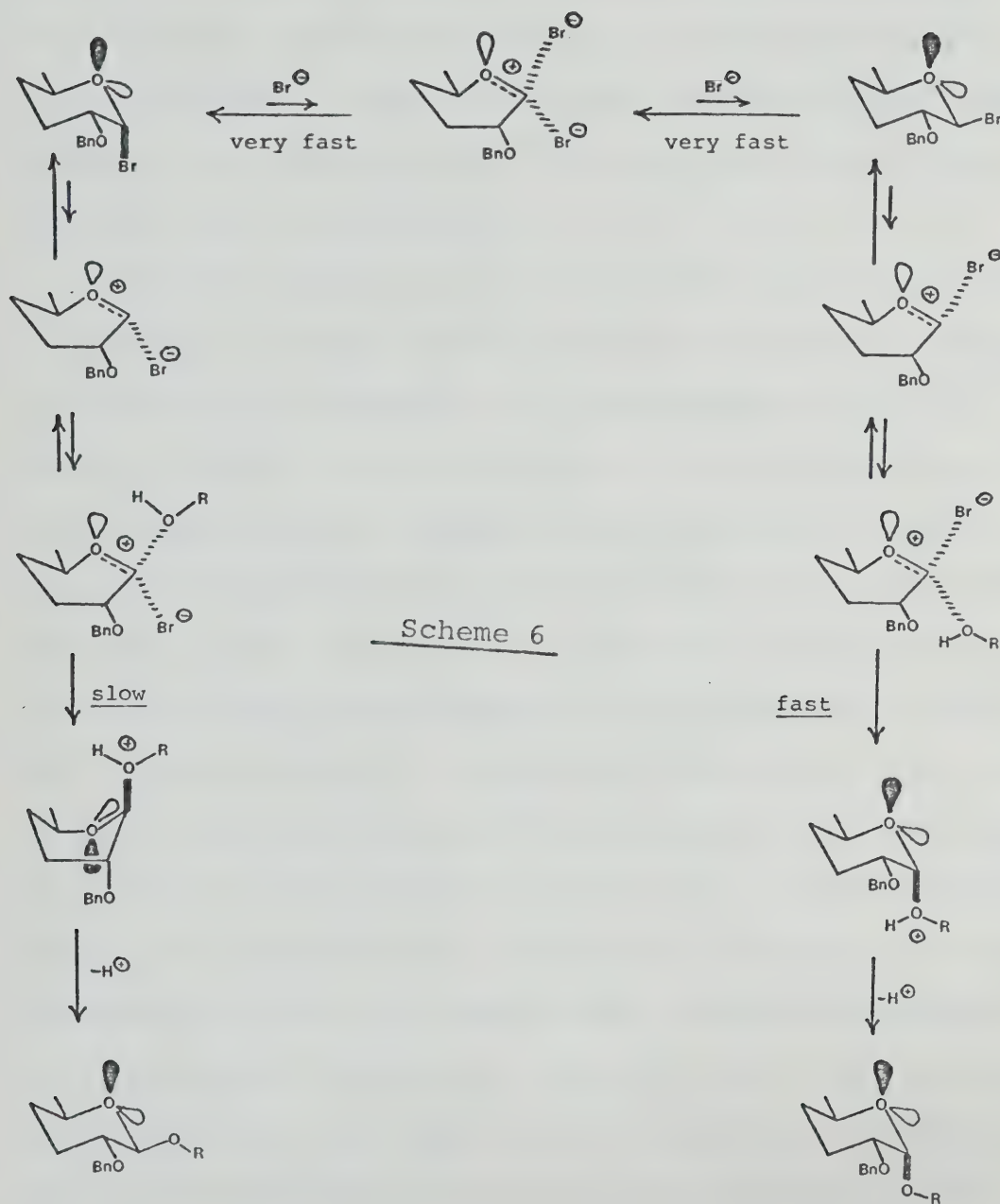
Scheme 5

The general strategy for the formation of the 1,2-trans- β -glycosidic linkage is then conceptually simple and will involve the reaction of a 2-O-acyl glycosyl halide with the alcohol, in the presence of a halophile, under conditions sufficiently acidic to effect the orthoester rearrangement. The sensitivity of this rearrangement to steric, electronic and stereoelectronic effects can be fully appreciated by referring to Scheme 5 where some recent results obtained by Flowers³⁷ are summarized. The main problem will involve the optimization of the reaction for the particular application at hand.

2. The 1,2-cis- α -glycosidic Linkage

a. The Halide-ion Catalyzed Reaction³⁸

The establishment of the 1,2-cis- α -glycosidic linkage is, in general, much more straightforward. Until very recently, the only method suited to the task was the halide-ion catalyzed α -glycosylation reaction developed by Lemieux and co-workers.³⁸ This method involves guiding the reaction between an appropriately blocked glycosyl halide and an alcohol under solvolytic conditions by way of the β -halide to form the α -glycoside (Scheme 6). Because of the anomeric effect, β -halides are thermodynamically much less stable than their corresponding α -anomers and, therefore, exist in very low concentration in an equilibrium mixture. The



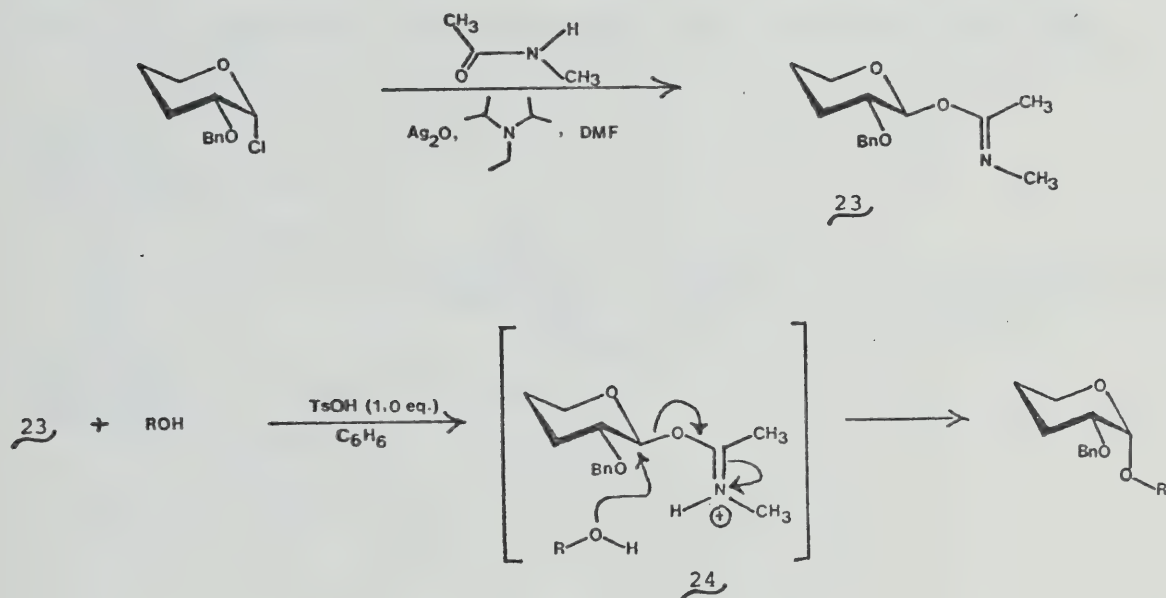
success of this reaction is attributed³⁸ to the much greater reactivity of the β -halide and the addition of added halide-ion, in the form of tetraalkylammonium halide, to ensure that a rapid equilibrium is established between the α and β halides. The reaction may then, in accord with Hammett-Curtin principles³⁹, be funnelled through the β -halide to the α -glycoside.

The high reactivity of β -halides compared with their α -anomers is thought to be based on the stereoelectronic demands for the formation of reactive intermediates³⁸. As seen in Scheme 6, the formation of α -glycoside from a glycosyl halide and an alcohol under solvolytic conditions is expected to proceed by way of ion pairs to alcohol triplets. The more rapid formation of α -glycoside is then attributed to a more ready decomposition of its precursor triplet than the triplet leading to β -glycoside formation. This situation has been rationalized³⁸ on the basis that the development of the glycosidic bond requires, in the transition state, an antiperiplanar arrangement between a lone pair of electrons on the ring oxygen atom and the developing bond. In the case of β -glycoside formation, this would require the achievement of a high-energy boat-like transition state while for α -glycoside formation the transition state would lead directly to the less strained 4C_1 chair conformation of the product.

The reaction usually employs perbenzylated glycosyl bromides as the glycosylating agents as the corresponding chlorides have proved to be inconveniently unreactive.³⁸ The choice of benzyl as blocking group was made primarily on the basis of the requirement of a non-participating protecting group at the 2-position. It might be expected, in addition, that pyranose ring substituents more electro-negative than benzyloxy might retard both the $\alpha \rightleftharpoons \beta$ anomerization of the glycosyl bromide and, likely more important, the alcoholysis of the β -bromide as these proceed through transition states with significant carbonium-ion character and without heavy-metal promotion. The α -glycosylation of an alcohol is generally carried out in dichloromethane as solvent in the presence of tetraethylammonium bromide, in an amount near-equivalent to that of the glycosyl bromide, and either Hünig's base⁴⁰ or molecular sieve 4Å to neutralize the hydrogen bromide that is produced. While these conditions frequently produce excellent yields of α -linked disaccharides, in more difficult cases the addition of dimethylformamide as co-solvent (ca. 20% in dichloromethane) was found⁴¹ to significantly increase the yields as well as the reaction rate. Yields in the order of 60-90% are regularly achieved even with highly complex saccharidic alcohols.

b. The Imidate Procedure^{42,43}

Recently a novel α -glycosylation reaction was developed by Sinaÿ and co-workers.^{42,43} This so-called 'imidate procedure', which is illustrated by the abbreviated formulas in Scheme 7, bears a conceptual resemblance to the halide-ion reaction in that α -glycoside formation proceeds through a

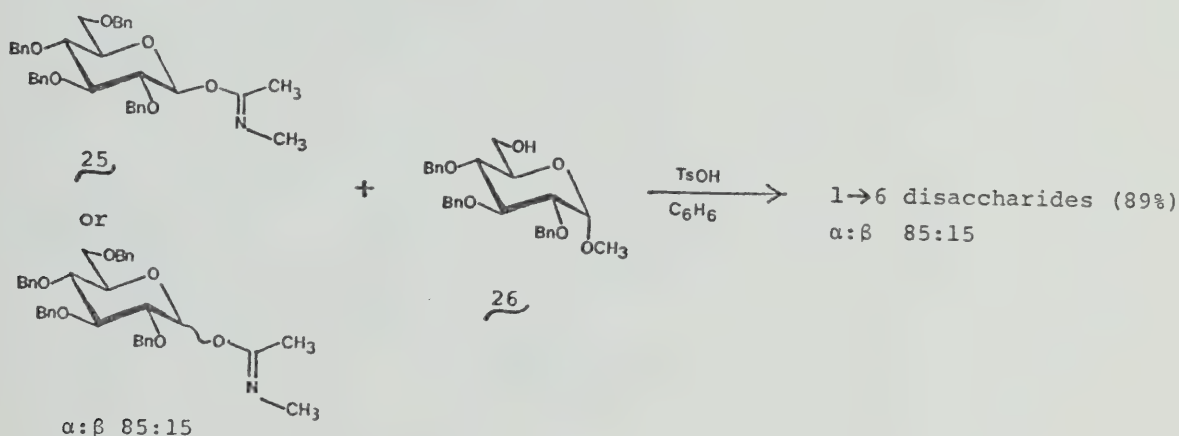


Scheme 7

double inversion at the anomeric center. The reaction is, however, distinct from the halide-ion reaction in that the β -imidate 23 is prepared separately and isolated, and is used as the glycosylating agent. The mechanism of this α -glycosylation, as suggested by the authors,⁴⁴ involves the protonation of the β -imidate 23 to provide the reactive

iminium salt 24, followed by an S_N^2 type of displacement by the alcohol to produce the α -glycosidic product.

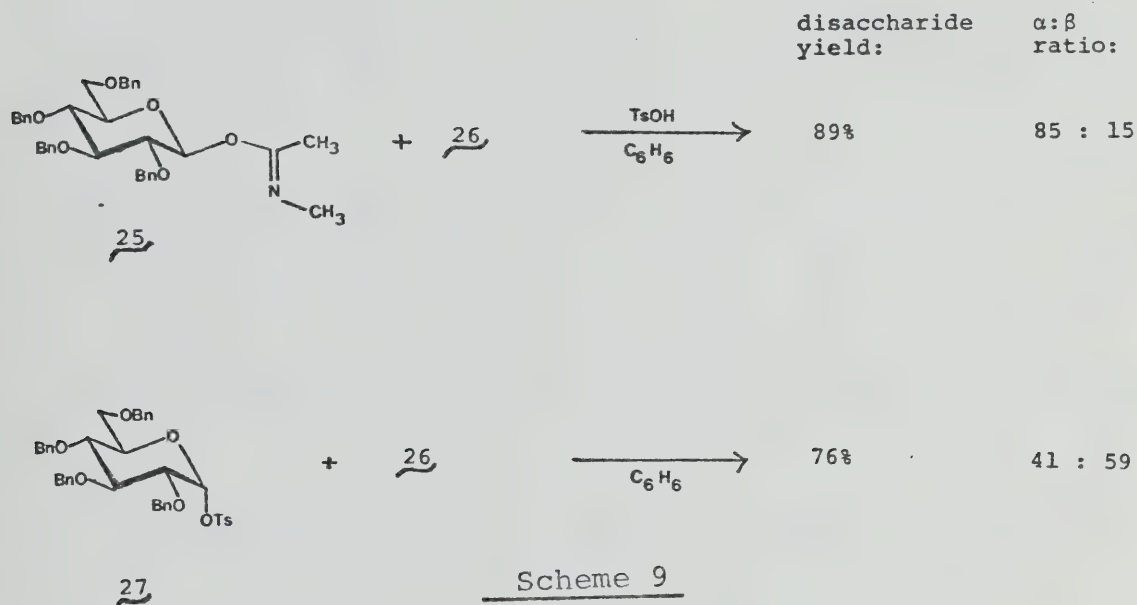
If the case were this simple, the authors would indeed be hard-pressed to explain some of their own results⁴⁵ (Scheme 8), since a mixture of imidate anomers ($\alpha:\beta = 85:15$) (produced from the β -imidate under essentially the same conditions as those for the glycosylation reaction) gave the



Scheme 8

same yield and anomeric ratio of disaccharides as was obtained with the pure β -imidate. The suggestion that the reaction may actually be proceeding through the intermediacy of a glycosyl sulfonate, produced by the reaction of the β -imidate 25 with the sulfonic acid, has been entertained.⁴⁵ The results obtained⁴⁵ in the condensation of

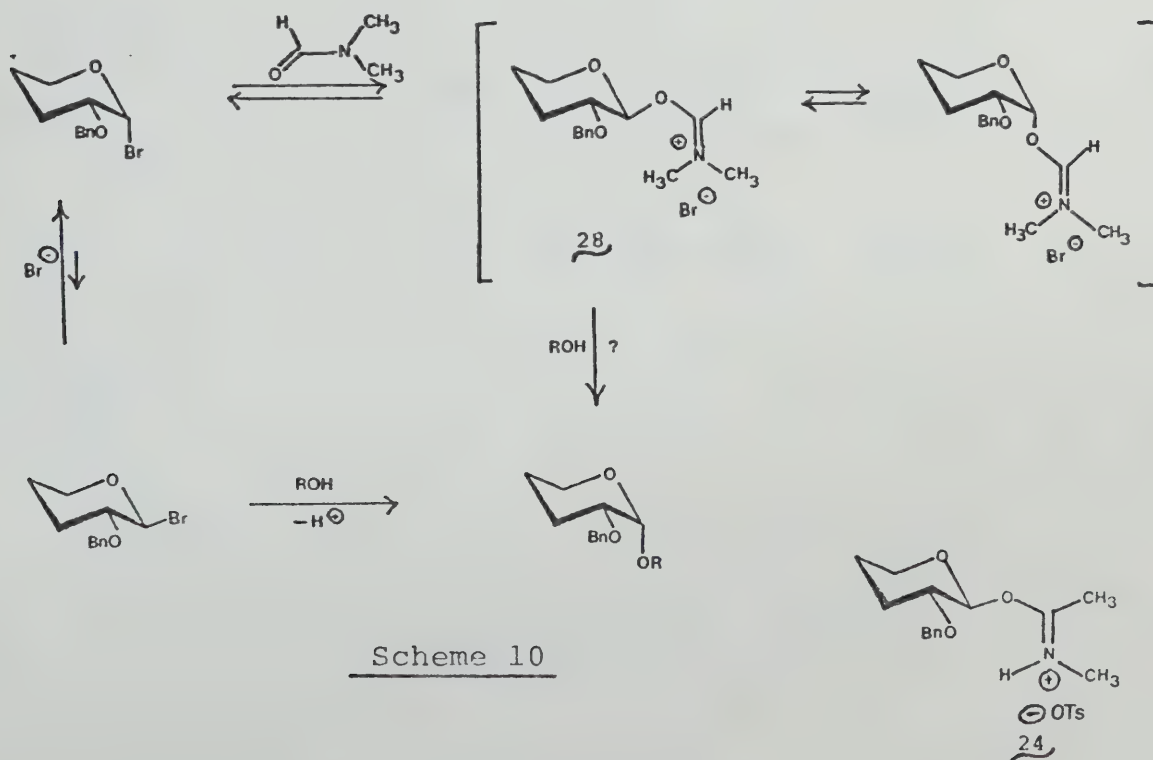
25 and 26, with *p*-toluenesulfonic acid as catalyst, are however clearly at variance with those obtained by Schuerch⁴⁶ when using the α -tosylate 27 in place of the imidate 25 (Scheme 9). Schuerch has also noted⁴⁷ that glycosylations



performed with anomeric triflates, in place of tosylates, generally result in a decrease of stereospecificity in the reaction. A similar loss of specificity was observed by Sinaÿ and Pougny⁴⁵ when using triflic acid, in place of *p*-toluenesulfonic acid, in the imidate procedure.

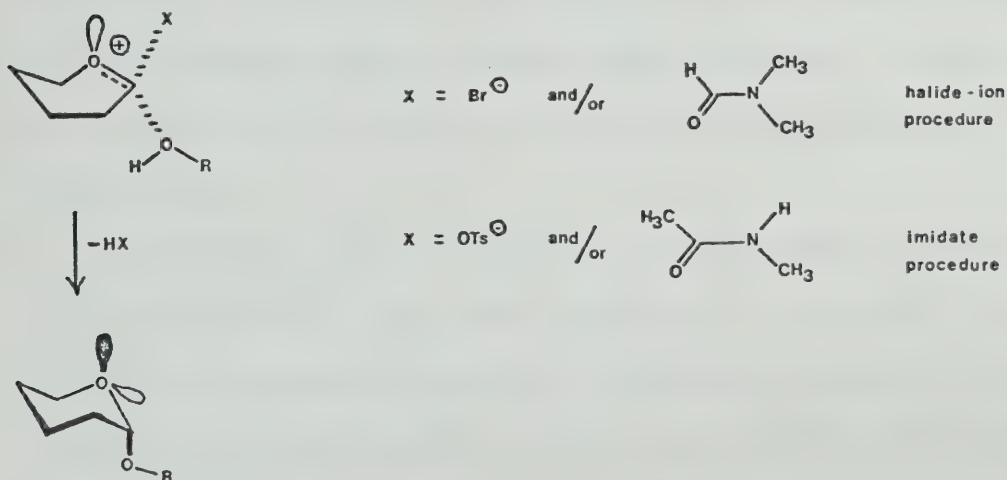
A rationale for the beneficial effects observed⁴¹ when using dimethylformamide (DMF) as a co-solvent in the halide-ion reaction may provide the common denominator to both of these α -glycosylation methods. A clue as to the role of

the DMF in this reaction may be obtained from an article⁴⁸ wherein the formation of the O-formylated derivative of the alcohol undergoing glycosylation was observed. Pozsgay⁴⁸ reported that attempted condensation of hepta-O-acetyl- α -D-kojibiosyl bromide with benzyl 2,3,4-tri-O-benzyl- β -D-glucopyranoside under halide-ion conditions (with DMF) produced no trisaccharide derivative, even after two weeks. Instead, a 70% yield of benzyl 2,3,4-tri-O-benzyl-6-O-formyl- β -D-glucopyranoside was formed. The formation of small amounts of O-formylated alcohols has also been observed, although infrequently, in these laboratories. This side reaction can only be rationalized as resulting from the in situ formation of a reactive anomeric iminium salt 28 which then acts, in some cases, as a Vilsmeier-type



formylating agent (Scheme 10). It can be appreciated that there is really only a trivial difference, in terms of structure, between the iminium salts 28 and 24; and, indeed, these may be regarded, in the limit, as solvated glycosyl carbonium ions.

It would seem most reasonable then to attribute the success of both the halide-ion reaction and the imidate procedure, as well as their various modifications, in the formation of the α -glycosidic linkage to the preferred rate of decomposition of the alcohol triplet leading to the α -glycoside (see Scheme 6); a situation which is favored for stereoelectronic reasons. The only real difference would then reside in the identity of the "X" part of the triplet (Scheme 11). There can be no doubt that the identity of



Scheme 11

"X" will affect the energetics of the reaction profile in a manner leading to different rates of reaction and, conceivably, to a very different product ratio of anomeric glycosides. However, in the case of both the imidate and halide-ion reactions, the nearly exclusive formation of α -glycosides requires these reactions to be under, or near, Hammett-Curtin control.

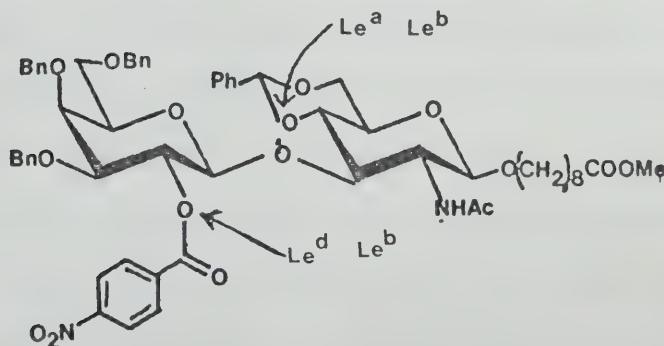
B. The Literature on Synthetic A, B, O(H) and Lewis-Active Oligosaccharides

Although the methods available for the formation of the glycosidic linkage remain far from perfect, particularly as regards the β -linkage, they have nevertheless permitted the preparation of a large number of synthetic oligosaccharides related to the A, B, O(H) and Lewis blood-groups. In the following discussion, the synthesis of a given determinant will be considered as accomplished whether it was synthesized as a reducing oligosaccharide or as a glycoside of the oligosaccharide.

The preparation of the A trisaccharide [α DGalNAc(1 \rightarrow 3)-[α LFuc(1 \rightarrow 2)] β DGal] has been reported by Lemieux⁴⁹ and David.⁵⁰ The B trisaccharide [α DGal(1 \rightarrow 3)[α LFuc(1 \rightarrow 2)] β DGal] has been synthesized in the laboratories of Lemieux,⁴¹ Sinay⁵¹ and Augé.⁵²

The preparation of the A and B (Type 1) and Lewis oligosaccharides required the formation of the β DGal(1 \rightarrow 3) β DGlcNAc linkage. Fortunately, the β -D-galactosylation of the

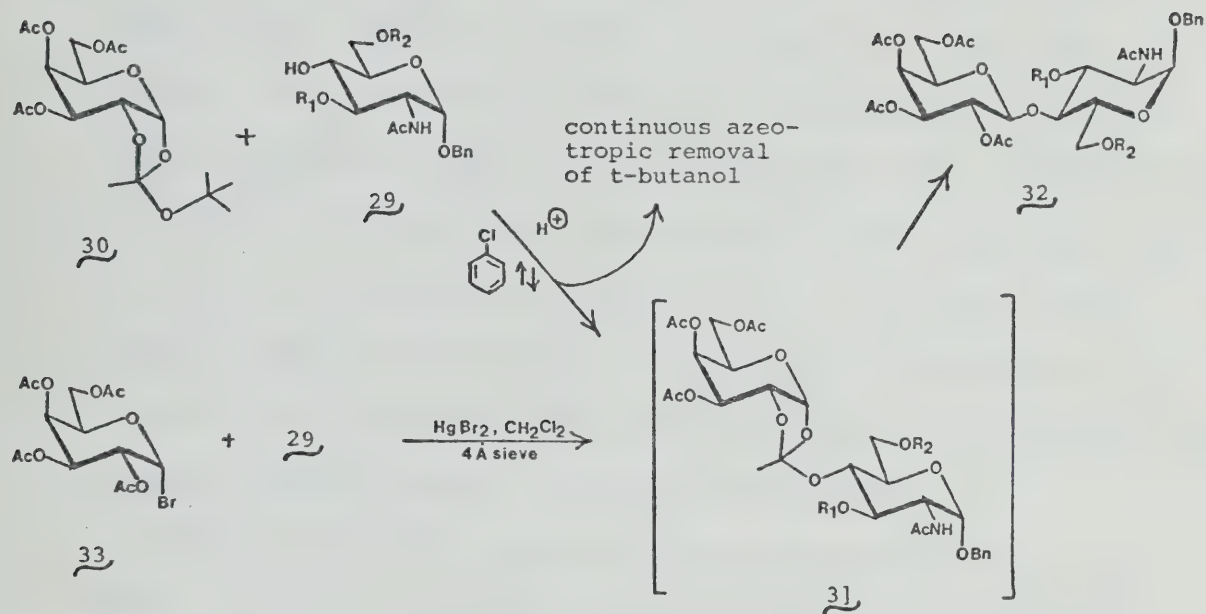
3-position of N-acetyl-D-glucosamine derivatives proceeds smoothly under a variety of conditions, hence the preparations of these oligosaccharides presented no particular difficulties. The synthesis of the Lewis-a trisaccharide [β DGal(1 \rightarrow 3)[α LFuc(1 \rightarrow 4)] β DGlcNAc] was first reported by Lemieux^{10,53} and later by Sinaÿ.⁵⁴ The Lewis-d trisaccharide [α LFuc(1 \rightarrow 2) β DGal(1 \rightarrow 3)] β DGlcNAc] has been prepared in the laboratories of both Lemieux¹⁴ and Paulsen.^{55,56} The Lewis-b tetrasaccharide [α LFuc(1 \rightarrow 2) β DGal(1 \rightarrow 3)[α LFuc(1 \rightarrow 4)] β DGlcNAc] was prepared by Lemieux, Bundle and Baker.¹⁴ A key intermediate used in the preparation of the Lewis-a, b and d oligosaccharides¹⁴ is shown in Scheme 12. The A and B (Type 1) tetrasaccharides [α DGalNAc(1 \rightarrow 3)] α LFuc(1 \rightarrow 2) β DGal(1 \rightarrow 3)] β DGlcNAc and [α DGal(1 \rightarrow 3)[α LFuc(1 \rightarrow 2)] β DGal(1 \rightarrow 3)] β DGlcNAc] have been synthesized by Lemieux⁵⁷ and Paulsen.^{55,56}



Scheme 12

The stepwise synthesis of the Type 2 determinants required, as the first step, the preparation of a suitably protected N-acetyl-D-lactosamine derivative. The development of a practical synthesis of N-acetyl-D-lactosamine had, in itself, been a long-standing problem. Koenigs-Knorr condensations between 2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl bromide (33) and 2-acetamido-3,6-di-O-acetyl-2-deoxy-D-glucopyranose derivatives, the most accessible derivatives of N-acetyl-D-glucosamine having the 4-hydroxyl group free, proceeded very poorly^{58,59}. Good yields of β -1 \rightarrow 4 linked products could only be realized when using acyclic⁶⁰ or 1,6-anhydro⁶¹ derivatives of N-acetyl-D-glucosamine. These problems were finally overcome by Sinaÿ⁶² in 1974.

What had until then been called "the lack of reactivity of the 4-hydroxyl group" of 2-acetamido-2-deoxy-D-glucopyranose can now be attributed not to any inherent lack of reactivity of this hydroxyl group but to the nature of the protecting groups being used in the preparation of the alcohol which leads to the aglycon. Using benzyl 2-acetamido-3,6-di-O-benzyl-2-deoxy- α -D-glucopyranoside (29a) as the alcohol and the orthoester 30 as the glycosylating agent, Sinaÿ⁶² was able to prepare the N-acetyl-D-lactosamine derivative 32a, by the orthoester method, in 75%

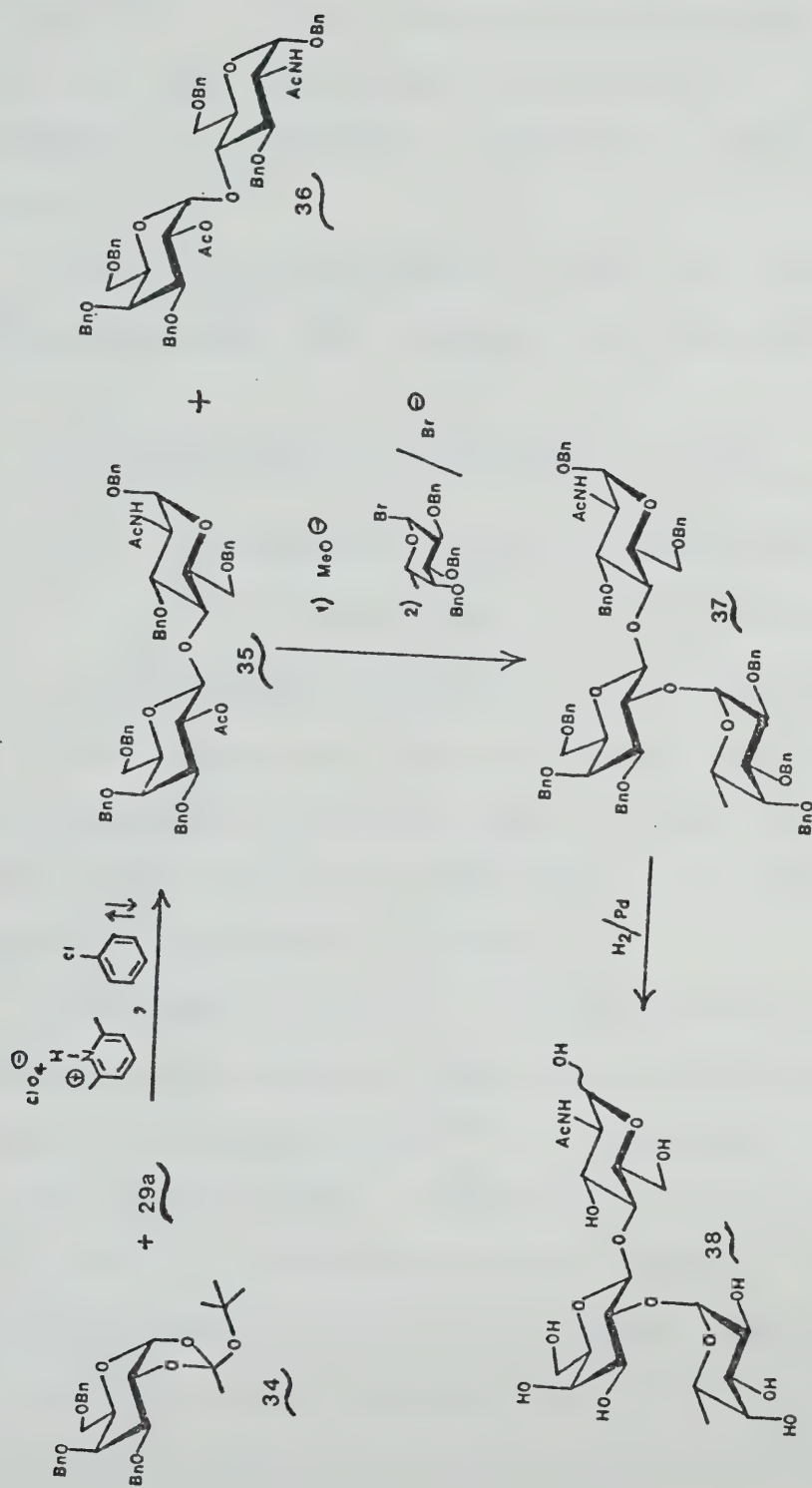


Glycosylating agent	alcohol	R ₁	R ₂	yield of 32
30	29a	Bn	Bn	75
33	29a	Bn	Bn	87
33	29b	allyl	Bn	77
33	29c	allyl	Ac	78
33	29d	Ac	Bn	5
33	29e	Ac	Ac	5

Scheme 13

yield (Scheme 13). The nature of the protecting groups in 29 was later shown by Sinaý⁴⁴ to be critical to the outcome of the glycosylation reaction performed under Helferich conditions, using 33, as well (Scheme 13). This is, of course, not surprising since both glycosylations are expected to proceed via the same orthoester 31. Examination of Scheme 13 shows that the success of the glycosylation reaction is dependent on the use of an ethereal, rather than an acetyl, protecting group at the 3-position of 29 and this well explains the earlier difficulties encountered in the preparation of N-acetyl-D-lactosamine.

With the major problem of the establishment of the Type 2 linkage resolved, the way to the H trisaccharide was clear. The synthetic route followed by Sinaý²² in the first reported synthesis of this compound is summarized in Scheme 14. The orthoester glycosylation method was again used for the establishment of the Type 2 linkage, but with the tri-O-benzyl orthoester 34, a mixture of the β (52%) and α (12%) disaccharide derivatives 35 and 36 was obtained, demonstrating once more the sensitivity of the orthoester rearrangement. Condensation of 34 with the 3-O-allyl derivative 29b also gave⁶³ a mixture of β (38%) and α (16%) 1 \rightarrow 4 linked disaccharide derivatives whereas only the β -linked product (77%) was detected when using the corresponding acetylated bromide 33. Deacetylation of 35



Scheme 14

followed by α -L-fucosylation under halide-ion conditions provided the blocked trisaccharide derivative 37 (80%) which was then deprotected to provide 38. The H trisaccharide has since been prepared in several laboratories.^{63,65,66}

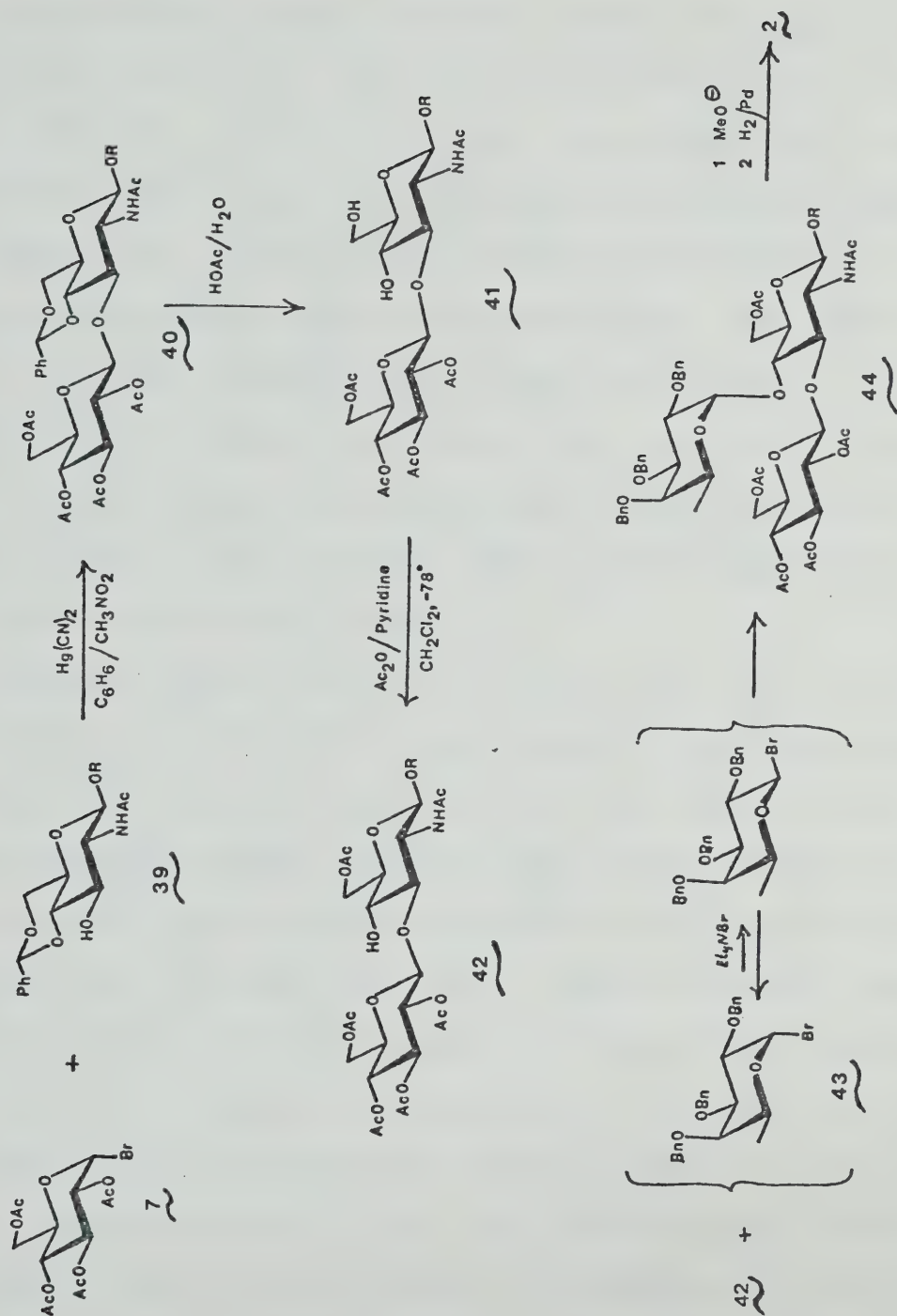
Finally, the syntheses of both the A and B (Type 2) tetrasaccharides have recently been reported^{65,66}.

C. Synthesis of the Trisaccharide Haptens.

1. 8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-4-O-(α -L-fucopyranosyl)-3-O-(β -D-glucopyranosyl)- β -D-glucopyranoside (2). The 4'-epi-Lewis-a Hapten.

The synthesis of the e-Le^a hapten followed essentially the procedure outlined by Lemieux, Bundle and Baker¹⁰ for their preparation of the Le^a hapten. The overall synthetic scheme is summarized in Scheme 15.

The starting material for the preparation of 2 was 8-methoxycarbonyloctyl 2-acetamido-4,6-O-benzylidene-2-deoxy- β -D-glucopyranoside¹⁰ (39) which was in ready supply in these laboratories. Condensation of 39 with 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide⁶⁷ (7) under Helferich conditions gave the β -linked product 40 in 77% yield. The anomeric configuration of the newly established glycosidic linkage in 40 was determined on the basis of its



Scheme 15

R = (CH₂)₈COOMe

¹Hnmr spectrum which is presented in Fig. 9.

The signals for the anomeric protons in 40 were expected in the region 4.5 - 5.0 ppm, along with those for H-2', H-3' and H-4'. It has consistently been observed, in the course of these ¹Hnmr investigations, that protons bound to carbon atoms bearing acetoxy groups have substantially longer longitudinal relaxation times (T_1 's) than those bound to carbon atoms bearing alkoxy substituents. This observation provides a ready means for simplifying the area of a spectrum where both these types of protons provide their signals. The 'anomeric region' in the spectrum of 40 presents just such a case.

Using a delay time of $\tau = 0.30$ sec. in the irradiation pulse sequence $180^\circ - \tau - 90^\circ$, this region of the spectrum simplified to the extent that H-2', H-3' and H-4' (of the peracetylated glucosyl residue) appeared as the only signals of negative intensity while the H-3 resonance had decayed to near zero intensity (Fig. 9c). The two anomeric doublets were now clearly visible as positive signals and the magnitude of their coupling constants ($J_{1,2} = J_{1',2'} = 8.0$ Hz) required⁶⁸ both glycosidic linkages to have the β -configuration. The assignment of the lower field doublet to H-1 was made in the following manner. Irradiation of the NH doublet of the acetamido group (δ 5.94) resulted in the collapse of the complex signal at δ 3.02 to a doublet

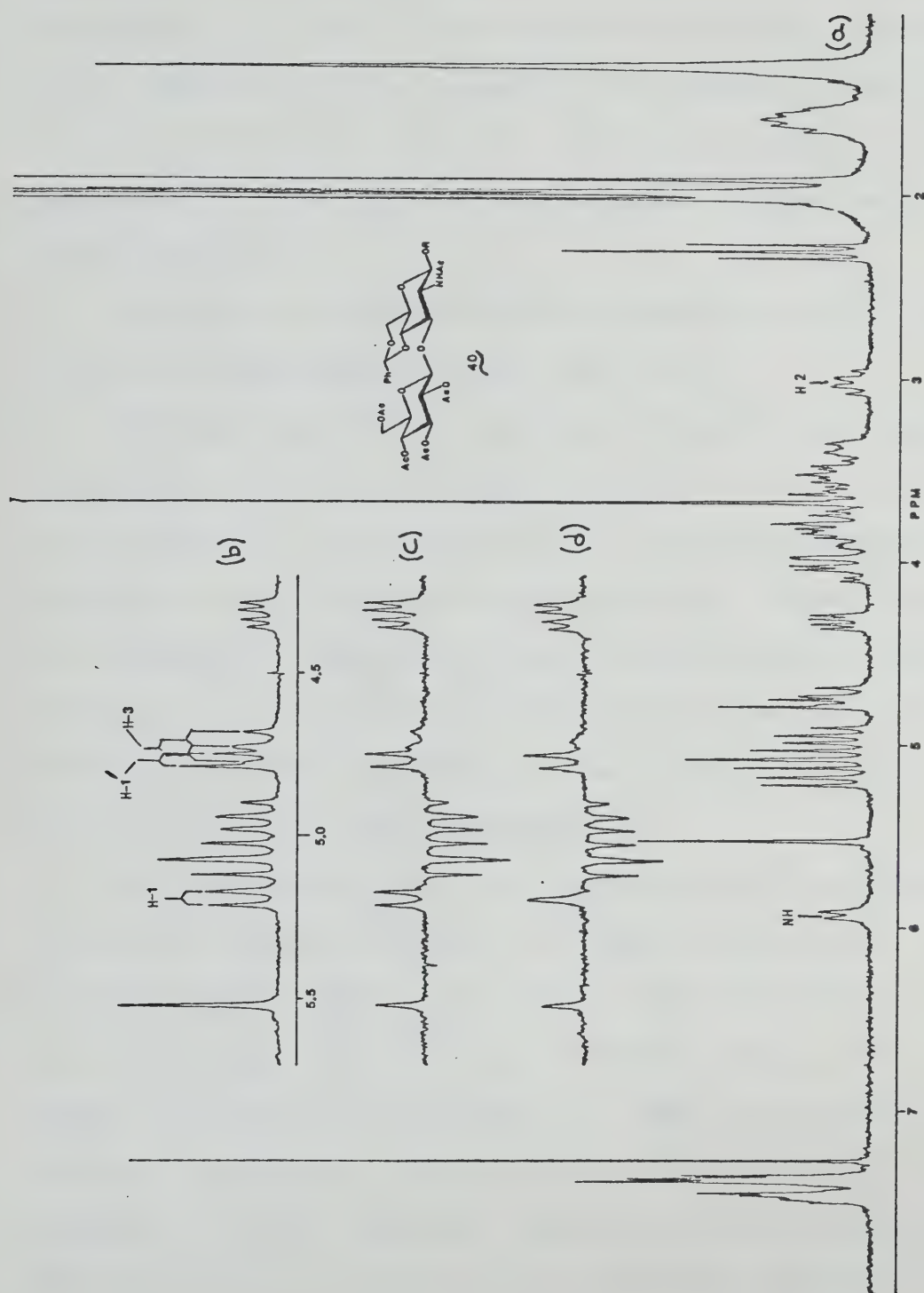


Fig. 9 The 200 MHz ^1H NMR spectrum of compound 40 in CDCl_3 : a, complete spectrum; b, expansion of the region 4.2 - 5.7 ppm; c, partially relaxed ($\tau = 0.30$ sec) spectrum; d, partially relaxed ($\tau = 0.30$ sec) observed while irradiating H-2 ($\delta 3.02$).

of doublets which must therefore be H-2. Irradiation of this signal, in turn, in the partially relaxed spectrum of 40 resulted in the collapse of the anomeric doublet at δ 5.21 to a singlet (Fig. 9d), thereby allowing its assignment as H-1. The higher field anomeric doublet would therefore be H-1'.

Removal of the benzylidene protecting group of 40 by mild acid hydrolysis provided the diol 41.

The next step in the synthesis required the specific protection of the 6-hydroxyl group of 41. In the synthesis of the Le^a trisaccharide (1),¹⁰ N-acetylimidazole was found to be an effective acetylating agent in this regard. For simplicity, however, 41 was acetylated using a 10% molar excess of acetyl chloride and pyridine in dichloromethane at -78°C ⁶⁹ providing a near quantitative yield of the 6, 2',3',4',6'-pentaacetate 42.

The position of the free hydroxyl group in 42 could be readily determined from its ¹Hnmr spectrum (Fig. 10) recorded using DMSO-d₆ as solvent. Since proton exchange is slow in this solvent,⁷⁰ the hydroxyl group proton will remain coupled to its γ -proton(s). The appearance of the hydroxyl proton as a doublet in the spectrum of 42 (Fig. 10) required it to be vicinally coupled to one proton only. The unlikely possibility that acetylation had occurred at the 4-position could therefore be discounted since the

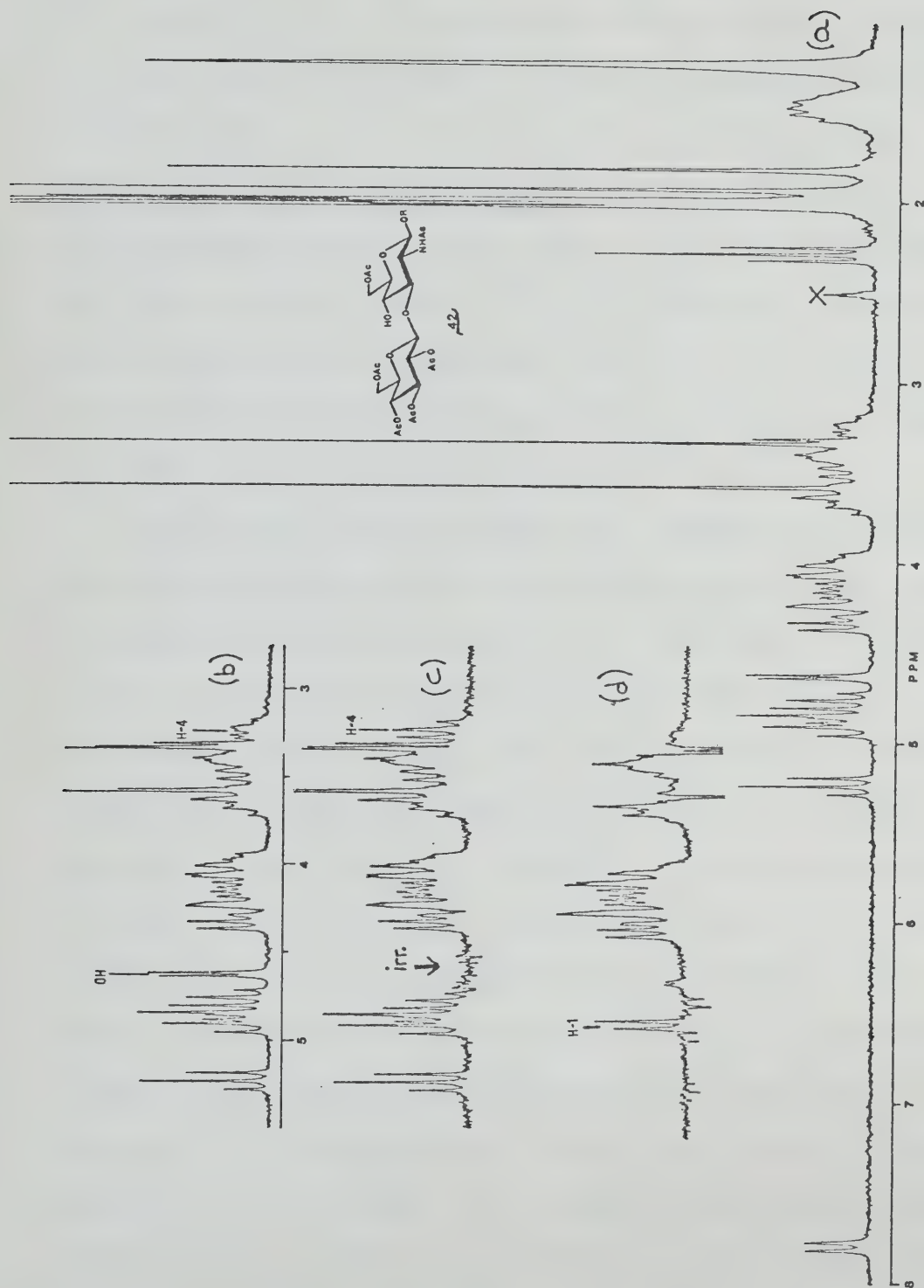


Fig. 10 The 200 MHz ^1H NMR spectrum of compound 42 in $\text{DMSO}-d_6$: a, complete spectrum; b, expansion of the central portion of the spectrum; c, spectrum observed while irradiating the OH resonance; d, partially relaxed ($\tau = 0.30$ sec) spectrum.

resulting primary hydroxyl group would have been coupled to the two H-6's. Irradiation of the hydroxyl group proton (δ 4.62) in the spectrum of 42 also collapsed the complex signal for H-4 (δ 3.22) to a well-resolved triplet (Fig. 10c). The usefulness of partially relaxed spectra is again demonstrated in Fig. 10d. Thus, while one of the signals for the anomeric protons of 42 is completely buried in the normal spectrum, the signals for H-2', H-3' and H-4' have virtually vanished in the partially relaxed spectrum leaving this signal clearly visible at δ 4.86.

Halide-ion catalysis for α -glycopyranoside formation³⁸ was employed to establish the α -L-fucopyranosyl linkage in the blocked trisaccharide 44. The reaction of 42 with 2,3,4-tri-O-benzyl- α -L-fucopyranosyl bromide⁷¹ (43) was performed in the presence of tetraethylammonium bromide to ensure rapid equilibration of 43 with its β -anomer since the halide ion catalyzed formation of the α -glycosidic bond is dependent on the more rapid reaction of the β -bromide with the alcohol.³⁸ Although the reaction was slow (6 days) and required a large excess of 43, it provided an essentially quantitative yield of 44. The ^1H and ^{13}C nmr spectra of 44 required the presence of a single anomer although, at this stage, the anomeric configuration of the fucosyl group could not be unequivocally established. Compound 44 was deacetylated and the product directly

debenzylated to provide the title trisaccharide.

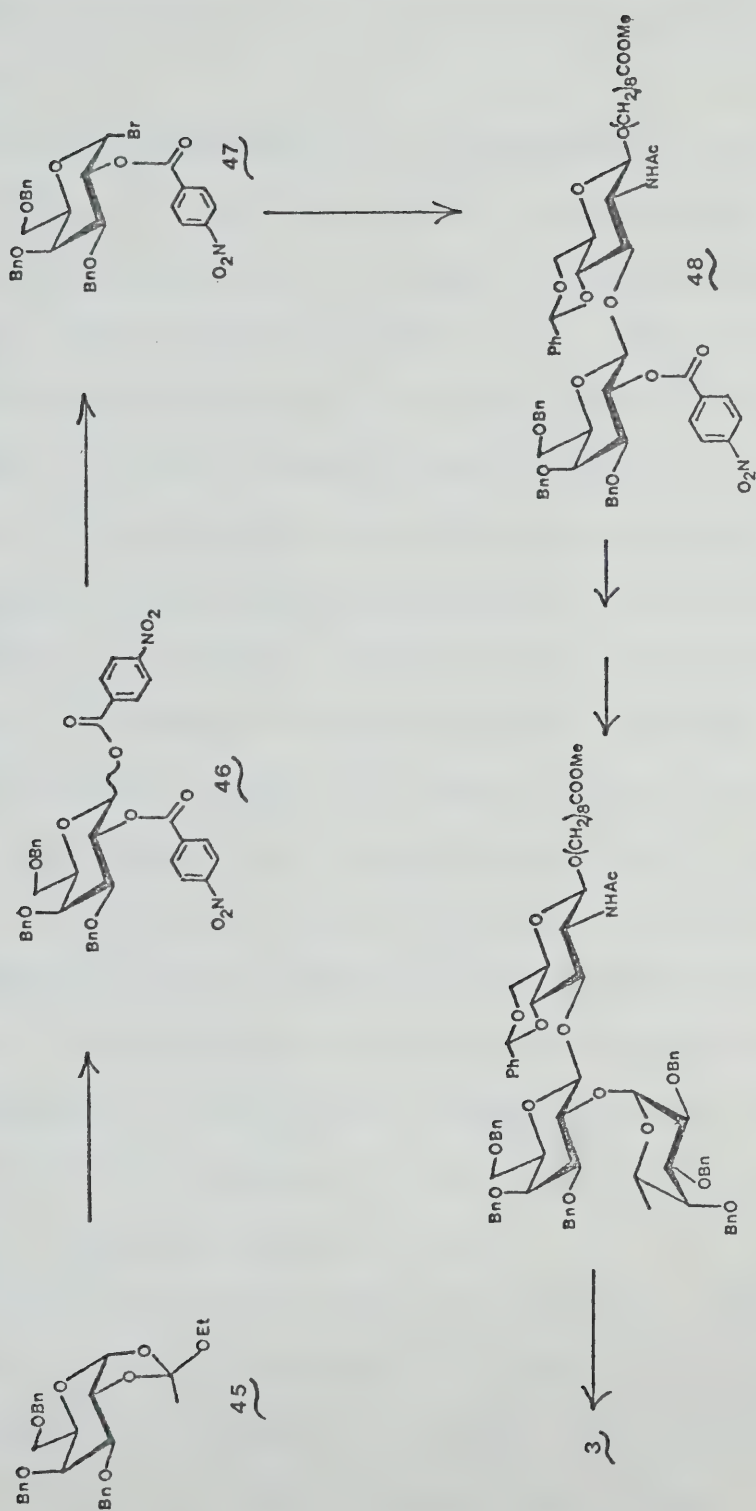
The ^1H and ^{13}C nmr spectral parameters for the e-Le^a trisaccharide (2) are presented in Tables 1 and 2 (Chapter 3). A coupling constant of 3.6 Hz for H-1' (Table 1) in 2 requires⁶⁸ the L-fucopyranosyl group to be present in the α -configuration, as expected.

The known proneness of acetyl groups to migration⁷² raised the possibility that fucosylation of 42 may have been preceded by acetyl migration from the 6 to the 4-position under the predominantly basic conditions of the halide-ion reaction. As Lemieux and Driguez⁵³ point out, it is well established⁷³ that the hydroxymethyl-group carbons of hexopyranosides provide their ^{13}C nmr signals in the region 60 to 63 ppm downfield from tetramethylsilane with deuterium oxide as solvent. Glycosylation of these hydroxyl groups causes a deshielding of 7 to 10 ppm of the corresponding carbons. Inspection of Table 2 shows the signals of the two hydroxymethyl carbons to be in this region, with the signal for C-6 at 60.58 ppm. The possibility that acetyl migration and subsequent 6-O-fucosylation had occurred could therefore be rejected.

2. 8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-3-O-
[2-O-(α -L-fucopyranosyl)- β -D-glucopyranosyl]- β -
D-glucopyranoside (4). The 4'-epi-Lewis-d Hapten.

The salient features of the route chosen by Lemieux and Baker¹⁴ for the synthesis of the Le^d hapten (3) are shown in Scheme 16. Experimental procedures, in addition to those presented in the patent, were available. Their strategy required the preparation of a suitably protected key intermediate, the galactosyl bromide 47, having a participating group at the 2-position that could be selectively removed after establishment of the β -glycosidic linkage in 48. Subsequent bromide-ion catalyzed α -L-fucosylation of the resulting alcohol, followed by deprotection, afforded the Le^d trisaccharide. The p-nitrobenzoyl ester was chosen as participating-protecting group in 47 in the hopes that 46 would be an easily purifiable crystalline compound. This, indeed, proved to be the case. Compound 46, in turn, was prepared by the sequential acid and base catalyzed hydrolysis of 45, followed by p-nitrobenzoylation of the resulting diol.

Following this general strategy, the first step in the synthesis of 4 was the preparation of the gluco-analogue of 45; namely, 3,4,6-tri-O-benzyl-1,2,0-[1-ethoxyethylidene]- α -D-glucopyranose (50). This compound was produced in 84%

Scheme 16

yield by deacetylation and benzylation of the readily accessible 3,4,6-tri-O-acetyl-1,2-O-[1-ethoxyethylidene]- α -D-glucopyranose.⁷⁴

Rather than paralleling the sequence of reactions shown in Scheme 16, it seemed desirable to establish a method for the direct conversion of 50 to the 2-O-acetylglucosyl halide, thus eliminating four steps in the overall synthesis. Several transformations of this type have been reported, the most useful of which seemed to be the reaction of the 1,2-O-alkoxyethylidene group with trimethylsilylchloride.⁷⁵ Trans-acetoxy-chlorides were produced in excellent yields. Glycopyranosyl chlorides, however, are known to exhibit low reactivity,¹⁷ notably in the Helferich reaction. The use of trimethylsilyl bromide for the equivalent transformation⁷⁶ was considered but its potential ability for the dealkylation of benzyl ethers⁷⁷ led to the rejection of its usage. Both hydrogen chloride⁷⁸ and hydrogen bromide⁷⁹ have been used to effect this transformation on peracetylated 1,2-orthoesters. When 50 was allowed to react with hydrogen bromide in dichloromethane at -35° , however, an intractable mixture of compounds was produced.

It could be expected that the orthoester 50 would be subject to ready acetolysis. A report,⁷⁶ in fact, existed where an orthoester to halide transformation was affected by both acetyl bromide and acetyl

chloride. With acetyl bromide as acetolyzing agent, the course of reaction shown in Fig. 11 might be anticipated. The first step would lead to the formation of ethyl acetate and the resonance-stabilized dioxolan-2-ylum ion pair 51. Collapse of this ion pair would be expected to lead to the β -bromide 52 which, in the presence of bromide ion, should anomerize to the α -bromide 53.

These expectations were borne out in all respects. The course of the reaction of 50 with acetyl bromide could be most conveniently followed by performing the reaction directly in an nmr probe. Addition of acetyl bromide (3 equivalents) to 50 dissolved in dry CD_2Cl_2 resulted in the rapid consumption of the orthoester, as evidenced by the decrease in the intensity of the signal for its methyl group at $\delta 1.62$. This signal had essentially disappeared after 20 minutes. After only 2 minutes, a new and readily observable doublet ($J_{1,2} = 8.5 \text{ Hz}$) at $\delta 5.50$ became apparent, whose intensity at first increased sharply with time and later fell back to zero. This doublet has been assigned to H-1 of the β -bromide 52. Unstable β -bromides have previously been prepared⁸⁰ and were found to rapidly equilibrate with the more stable α anomers. After 13 minutes, another doublet ($J_{1,2} = 4.0 \text{ Hz}$) started to appear at $\delta 6.63$ and increased in intensity throughout the remainder of the reaction. This signal has been assigned to H-1 of the

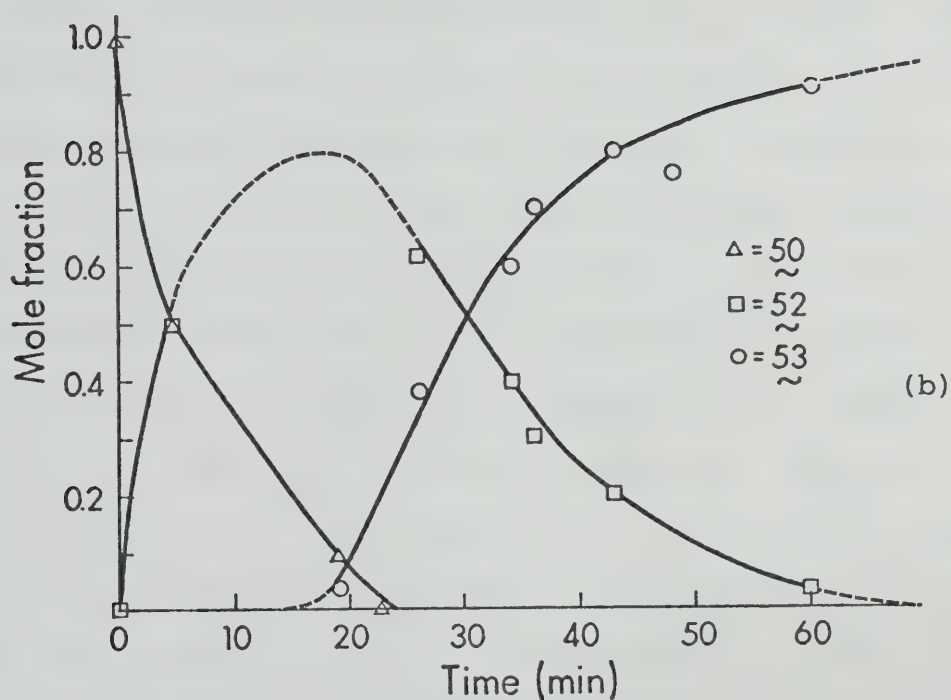
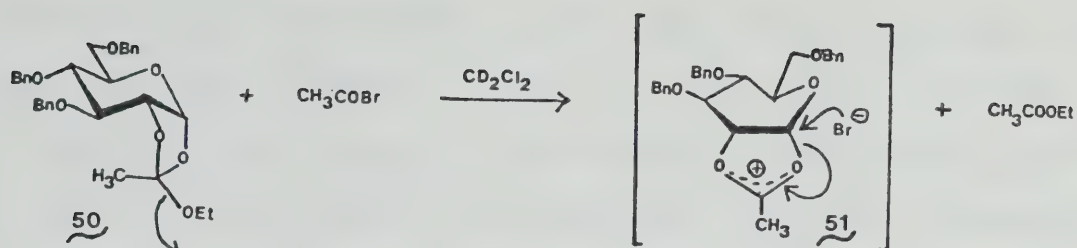
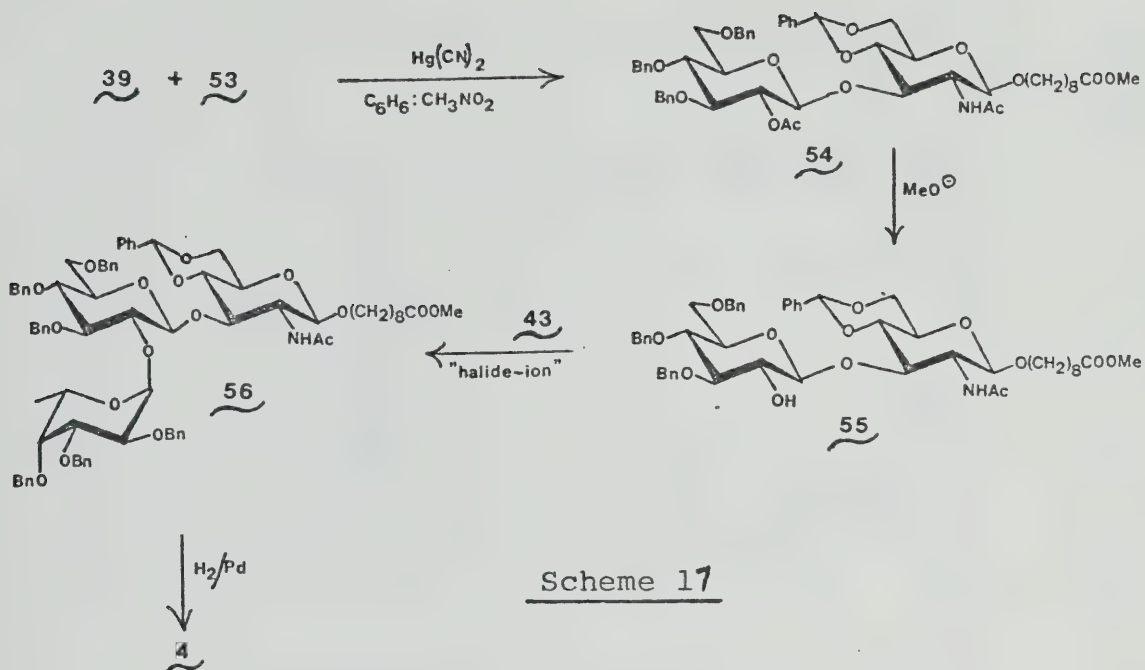


Fig. 11 The reaction of the orthoester **50** with acetyl bromide: a, anticipated course of reaction; b, kinetics of the reaction at 40°C in CD_2Cl_2 . The mole fractions of **50**, **52** and **53** were calculated from the relative intensities of their characteristic signals in the 60 MHz ^1H nmr spectrum of the reacting mixture.

α -bromide 53. The changes in the intensities of these signals with time is shown in Fig. 11. It is clear from Fig. 11 that the reaction of 50 with acetyl bromide leads initially to the formation of the β -bromide 52 which, subsequently, anomerizes at a slower rate to the α -bromide 53. The $^1\text{Hnmr}$ of the crude product isolated after 90 mins. of reaction confirmed the formation of 53 in approximately 90% yield.

For preparative purposes, the reaction was modified slightly. It was decided to add tetraethylammonium bromide to ensure the rapid anomerization of the β -bromide, thus eliminating the potentially troublesome hydrolysis of this reactive species during aqueous work-up. Molecular sieve 4\AA was also included, not only to ensure near anhydrous conditions in the larger scale reaction, but also to absorb the hydrogen bromide that would inevitably be present either in the acetyl bromide or as a result of its hydrolysis by traces of water in the reaction mixture. Hydrogen bromide is well known⁸¹ for its ability to debenzylate benzyl ethers in carbohydrate chemistry, particularly at temperatures as elevated as 25°C . In this manner, 53 could be isolated in essentially quantitative yield after reaction for 1 hour at room temperature.

The remaining steps in the preparation of 4 are summarized in Scheme 17. Condensation of 39 and 53 provided the



Scheme 17

β -linked product 54 in 57% yield. The anomeric configuration of the glucosidic linkage in 54 could be assigned on the basis of its $^1\text{Hnmr}$ spectrum, presented in Fig. 12.

The 'anomeric region' in this spectrum is partially obscured by the complex AB signal patterns of the benzyl groups in 54. The assignment of the doublet ($J_{1,2} = 8.0 \text{ Hz}$) at $\delta 5.20$ to H-1 was straightforward following the selective decoupling of the NH and H-2 protons as previously described. Since the 2'-hydroxyl group of 54 was protected as its acetate, it could be expected that H-2' would have a substantially longer T_1 than the other protons providing

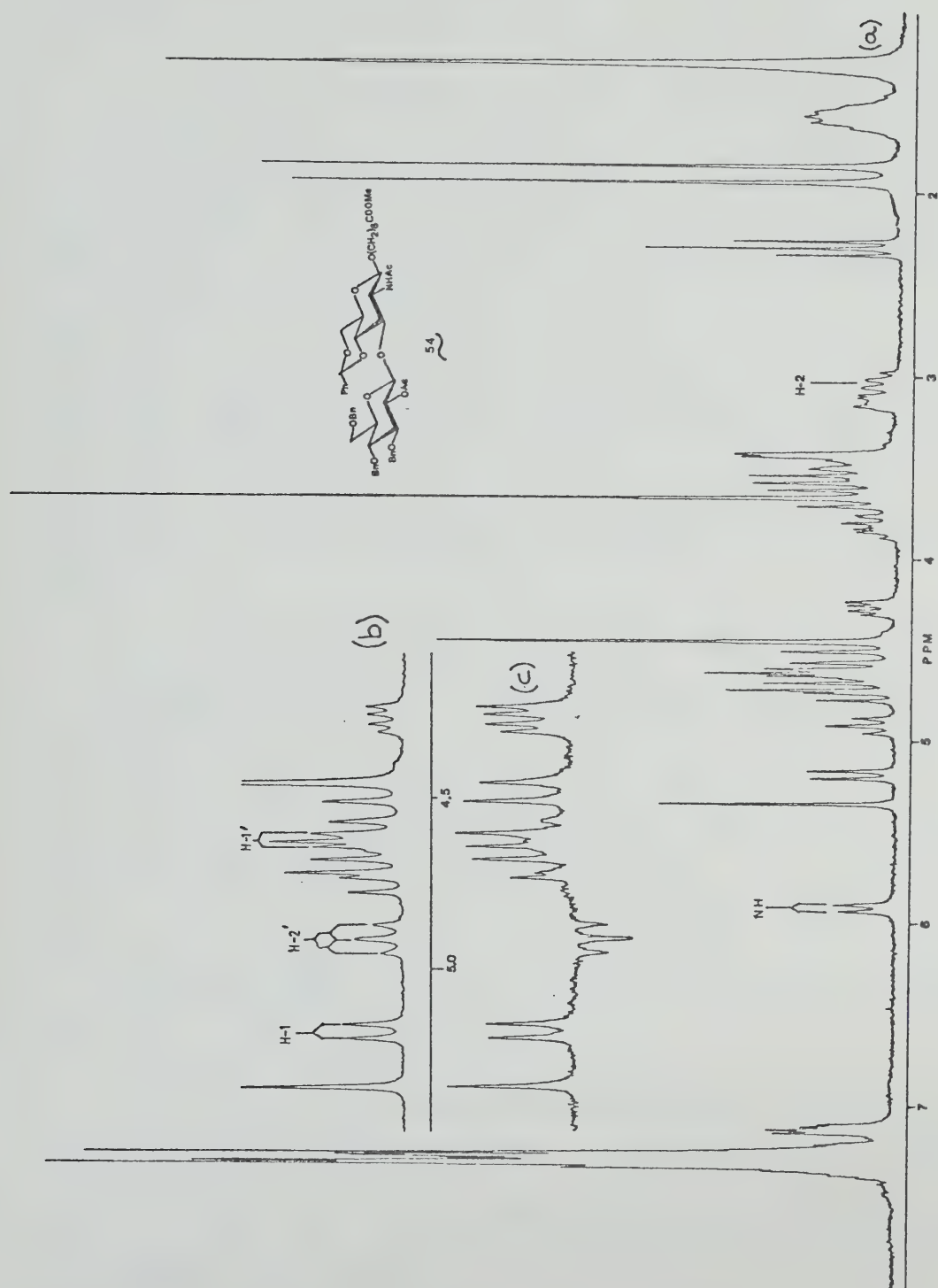


Fig. 12 The 200 MHz ^1H NMR spectrum of compound 54 in CDCl_3 : a, complete spectrum; b, expansion of the region 4.0 - 5.5 ppm; c, partially relaxed ($\tau = 0.30$ sec). spectrum.

their signals in this region. Indeed, the partially relaxed spectrum of 54 (Fig. 12c) showed only one negative signal ($\delta 4.93$, t, $J_{1',2'} \approx J_{2',3'} \approx 8.5$ Hz) which could therefore be assigned to H-2'. By comparison of the normal and partially relaxed spectra shown in Fig. 12, it is apparent that there can also be smaller but useful difference in T_1 's between the anomeric and benzylic protons. The signals corresponding to 4 of the 6 benzylic protons have virtually been eliminated in the partially relaxed spectrum allowing the doublet at $\delta 4.64$ to be assigned to H-1'. Irradiation of H-2' caused the collapse of this signal to a singlet.

Deacetylation of 54 provided 55. The α -L-fucosylation of 55 proved difficult to monitor by TLC and the reaction was interrupted after 41 hours. Column chromatography permitted the isolation of unreacted 55 (38%) and the blocked trisaccharide derivative 56 in 37% yield. No attempt was made to improve the yield of this reaction. Hydrogenolysis of the benzylidene and benzyl protecting groups in 56 provided the e-Le^d trisaccharide 4.

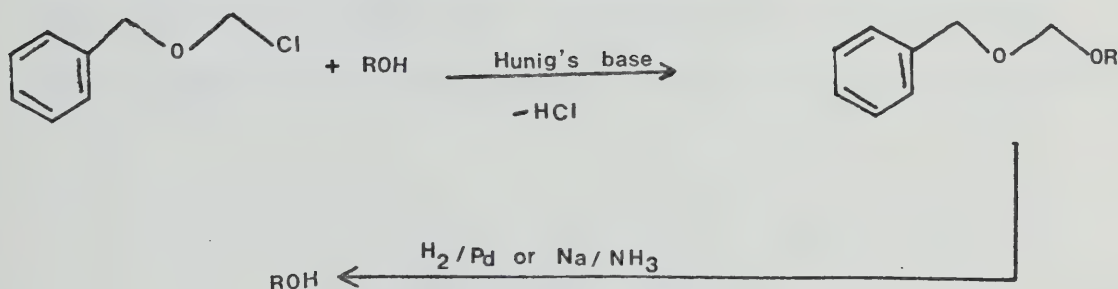
The ^1H and ^{13}C nmr spectral parameters for the e-Le^d trisaccharide (4) are presented in Tables 1 and 2, respectively (Chapter 3). These parameters were in accord with the assigned structure.

3. 8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-4-O-
[2-O-(α -L-fucopyranosyl)- β -D-galactopyranosyl]-
 β -D-glucopyranoside (5). The H Hapten.

At the time when this project was undertaken, the reducing form of the H-trisaccharide had already been prepared by Jacquinet and Sinay²². It was clear from their earlier work⁴⁴ on the synthesis of N-acetyl-D-lactosamine that good yields of β 1 \rightarrow 4 linked disaccharides could only be obtained when the 3-position of 2-acetamido-2-deoxy-D-glucopyranosides was blocked by an ethereal protecting group (recall Scheme 13). Indeed, all the published syntheses of the Type 2 linkage have since employed benzyl 2-acetamido-3,6-di-O-benzyl-2-deoxy- α -D-glucopyranoside (29a) as the aglyconic alcohol. Although success was virtually assured with the use of a glucosamine derivative protected in this manner, there was judged to be a pressing need for an ethereal protecting group that, unlike the benzyl or allyl groups, could be attached under near neutral conditions. Such a blocking group would allow the protection of partially acylated substrates without the prospect of deacylation or acyl-group migration. This protecting group would be particularly attractive if it could be removed under the hydrolytic conditions used for debenzylation and, ideally, this group might be selectively removed in the presence of benzyl ethers. To be compatible with the established

blocking/deblocking methodologies being used in oligo-saccharide synthesis, this protecting group should, in addition, be stable to mildly acidic conditions such as those required for the hydrolysis of the benzylidene group.

The only alcohol protecting group that was likely to meet these rather stringent requirements seemed to be the benzyloxymethyl group which had been in use in the laboratories of Stork⁸² and later Still.⁸³ In addition to the methods already described⁸² for the introduction and removal of this protecting group (Scheme 18), other conditions that

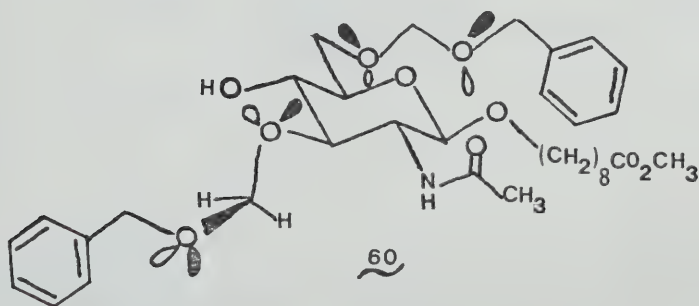


Scheme 18

could effect these same transformations could easily be envisaged. Following the work by Corey⁸⁴ on the selective cleavage of the methoxyethoxymethyl ether protecting group,

it might be expected that Lewis-acidic conditions could be found that would selectively remove the benzyloxymethyl group in the presence of benzyl ethers. Indeed, such conditions ($\text{TiCl}_4/\text{CH}_2\text{Cl}_2$, 25° , 5 min.) have now been established in these laboratories.⁸⁵

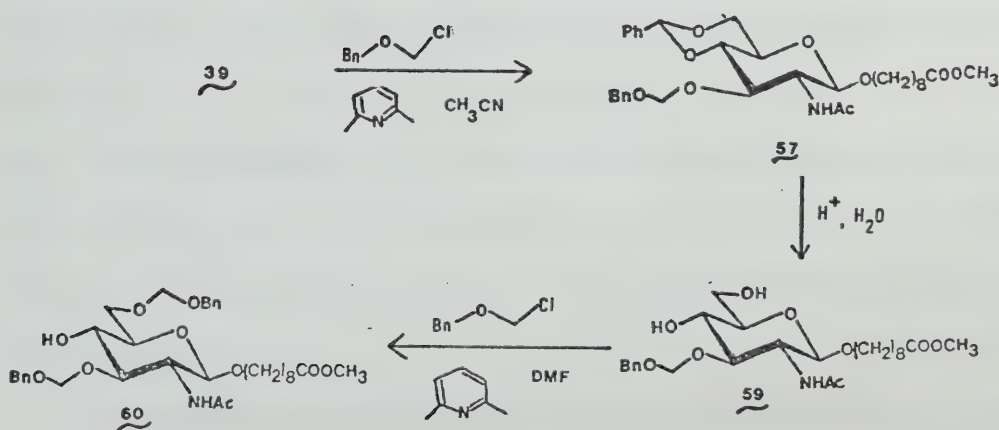
The first objective in the synthesis of 5 was then the preparation of 8-methoxycarbonyloctyl 2-acetamido-3,6-di-O-benzyloxymethyl-2-deoxy- β -D-glucopyranoside (60). Unlike the benzyl or allyl ethers, the benzyloxymethyl ether is expected to have certain distinct conformational preferences, notably about the acetal linkage. As a consequence of the anomeric effect, the allowed conformations for the benzyloxymethyl grouping are strongly limited to those in which a lone-pair of electrons on each of the oxygen atoms is antiperiplanar to a C-O bond in the acetal



Scheme 19

linkage. There should, however, be free rotation about the C-3 \rightarrow O-3 bond in 60 and it was apparent from the inspection of a molecular model that the benzyloxymethyl group could easily adopt a conformation, such as that shown in Scheme 19, where this grouping was well removed from the 4-hydroxyl group which was to be glycosylated. These same considerations also extend to the 6-O-benzyloxymethyl group of 60. It was thus hoped that, as was the case for the benzyl and allyl ethers, the presence of a benzyloxymethyl group at the 3-position of 60 would not sterically interfere with the glycosylation of the 4-hydroxyl group.

The synthetic route selected for the preparation of 60 is shown in Scheme 20.



Scheme 20

Reaction of 39 with benzyl chloromethyl ether,⁸⁶ in the presence of an excess of the hindered base, 2,6-lutidine, in acetonitrile at 80°C overnight provided the 3-O-benzyl-oxymethyl derivative 57 as a crystalline compound in 79% yield. Hydrogenolysis of a sample of 57, at this stage, in the presence of 10% palladium on carbon provided the known¹⁰ 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside 58 in quantitative yield, thus confirming that this protecting group could be removed under the standard conditions for both debenzylidenation and debenzylation. Compound 58 was identified by comparison with an authentic sample.

The benzylidene group of 57 could be selectively removed by acid hydrolysis using an aqueous acetic acid-dioxane mixture and provided, after chromatography, an 80% yield of the diol 59. Even these mild conditions, however, resulted in significant cleavage of the benzyloxymethyl group as a 12% yield of 58 was also isolated from the column. Nevertheless, this procedure did provide a useful yield of 59, and no attempt was therefore made to find conditions that would improve on the selectivity of the hydrolysis reaction.

It could be anticipated that benzyloxymethylation of the diol 59 would tend to favor the primary 6-position rather than the much more hindered 4-position. Indeed,

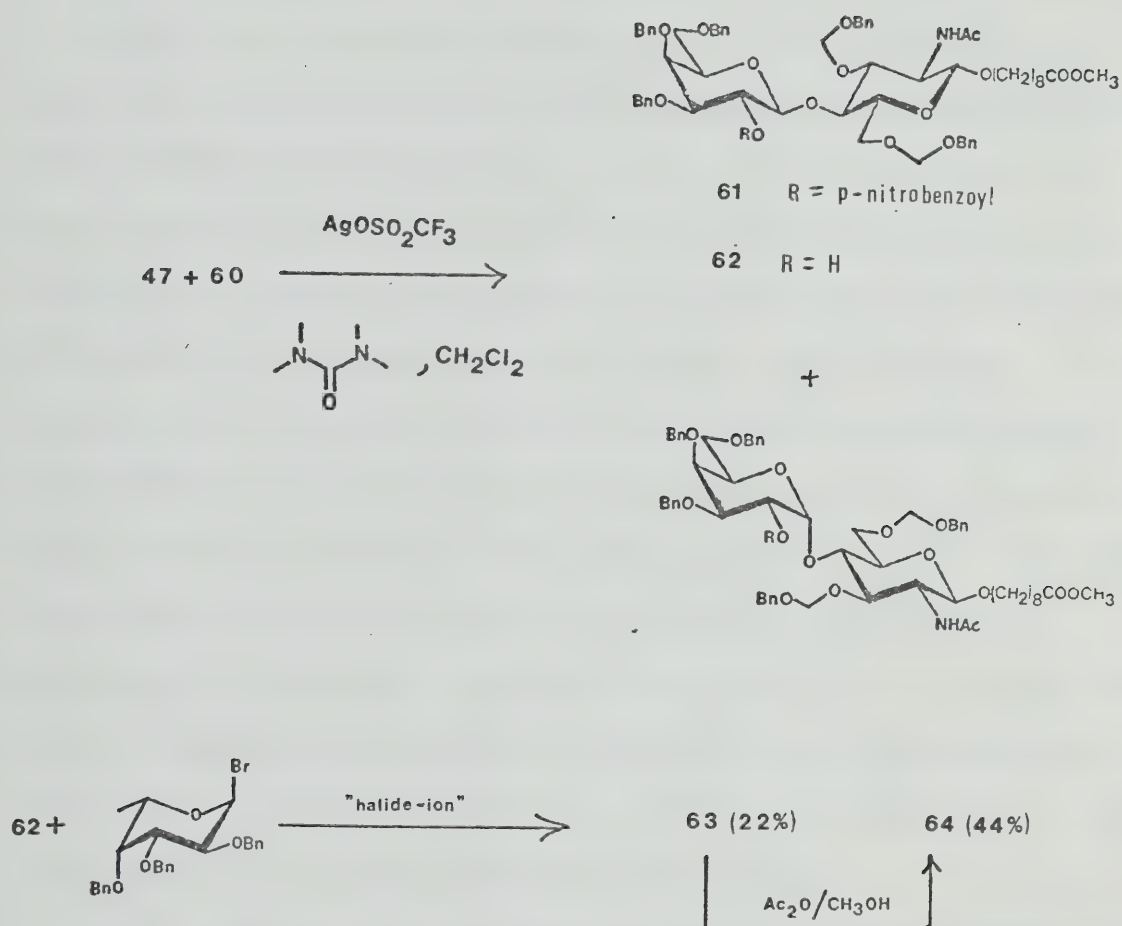
reaction of 59 with a 10% molar excess of benzyl chloromethyl ether gave a crude product whose thin layer chromatogram was consistent with the presence of unreacted 59 and the formation of only one new compound. The $^{13}\text{Cnmr}$ spectrum of this material clearly established the position of alkylation. A 4.33 ppm downfield displacement of the signal from C-6 of 60, when compared to that of 59, required⁷³ the benzyloxymethylation to have occurred at this position. Crystallization from aqueous ethanol provided pure 60 in 61% yield. An additional 9% could be obtained by chromatography of the mother liquor.

With a supply of the alcohol 60 in hand, attention was turned to its β -D-galactosylation. The good yields reported⁶⁵ in the glycosylation of 29a under Helferich conditions suggested the use of this method. A supply of 3,4,6-tri-O-benzyl-1,2-di-O-p-nitrobenzoyl- α,β -D-galactopyranose (46) was available and, as the corresponding bromide 47 had proved to be a successful glycosylating agent in the synthesis of the Le^{d} hapten,¹⁴ it was converted to 47 by reaction with hydrogen bromide in dichloromethane.¹⁴

Attempted condensation of 47 and 60 under Helferich conditions, however, gave no detectable disaccharide product. It was suspected that the p-nitrobenzoyl group might be responsible for the poor reactivity of 47, but the reaction of the corresponding peracetylated galactosyl bromide

33 with 60 under the same conditions again failed to provide any detectable disaccharide product. Evidently, the required orthoester rearrangement was being adversely affected by the benzyloxymethyl groups and different acidic conditions were required for success.

Following its initial use by Kronzer and Schuerch⁸⁷ it has been demonstrated^{35,36,88} that silver trifluoromethanesulfonate (triflate), in conjunction with an appropriate proton acceptor, is an excellent promotor of glycosylation between alcohols and glycosyl halides. When the condensation of 47 and 60 was conducted under the conditions of Hanessian and Banoub,⁸⁹ using tetramethylurea as proton acceptor, an approximately 60% yield of a mixture of disaccharide derivatives was obtained. The desired β -linked product 61 (Scheme 21) was isolated in 36% yield. The β -galactosyl configuration could be assigned to 61 on the basis of its $^1\text{Hnmr}$ spectrum where the signal for H-2' appeared as a doublet of doublets ($J_{1',2'} = 8.0 \text{ Hz}$, $J_{2',3'} = 9.5 \text{ Hz}$) at $\delta 5.54$. The other disaccharide product could be isolated in 21% yield (based on 60) after denitrobenzylation and chromatography. The small coupling constant (3.5 Hz) found for the anomeric proton of the galactosyl unit in the $^1\text{Hnmr}$ spectrum of this compound allowed its identification as the α -linked disaccharide derivative. Removal of the p-nitrobenzyl ester in 61



Scheme 21

provided the alcohol 62.

The α -L-fucosylation of 62, under the halide-ion conditions, proved to be the most entertaining of the reactions carried out during the course of this work. Reaction of 62 with the fucosyl bromide (43) (2.6 equivalents) was very rapid, as evidenced by TLC, with all the starting alcohol having been consumed within 2 hours. The formation of a major product (R_f 0.30 in n-hexane-ethyl acetate, 2:1), in addition to several other minor products with both greater and lesser chromatographic mobilities, was indicated. After 26 hours (the TLC had remained essentially unchanged) the mixture was worked-up and purified by column chromatography on silica gel using the same solvent system. All the components visualized on the thin-layer chromatogram appeared in the eluate except that of R_f 0.30. Furthermore, none of these minor products contained the methoxycarbonyloctyl grouping of the starting alcohol, as evidenced by the ^1H nmr spectra of the individual fractions.

The column was then washed out with a large volume of dichloromethane-ethylacetate (1:1). Evaporation of this eluate provided a chromatographically homogeneous material (ca. 88%) whose ^1H and ^{13}C nmr spectra clearly showed the presence of all three sugar units, but as a mixture of compounds. A solvent system was eventually found that could separate this material into its two components, which

have been designated as compounds 63 and 64. The $^1\text{Hnmr}$ spectra of these two trisaccharides are presented in Figs. 13 and 14, respectively. Examination of these spectra immediately showed that the fucopyranosyl residue was present in the α -configuration in both compounds on the basis of the small (ca. 3.5 Hz) coupling constant of its anomeric proton, eliminating the possibility of an anomeric mixture. Equally evident was the absence of resonances attributable to either the NH or COCH_3 protons of the acetamido group in 63, while these were present in the spectrum of 64. An 'anomalously' high field signal ($\delta 2.80$), integrating for one proton, was also present in the spectrum of 63 (Fig. 13). Examination of the $^{13}\text{Cnmr}$ spectra of these two compounds, shown in Figs. 15 and 16, confirmed the absence of the $-\text{COCH}_3$ grouping in 63 while both the carbonyl (170.12 ppm) and methyl (23.40 ppm) carbons were present in 64. Examination of the 'anomeric region' (95-105 ppm) showed the presence of five signals for both compounds, attributable to the three glycosidic carbons and the two O-methylenic carbons of the benzyloxymethyl groups.

It thus seemed probable that 63 was simply the N-deacetylated derivative of the expected trisaccharide 64. The small downfield shift of C-2 in 63, when compared to that in 64, was supportive of this conclusion.⁷ Irradiation of the doublet of doublets ($J_{1,2} = 8.0 \text{ Hz}$, $J_{2,3} =$

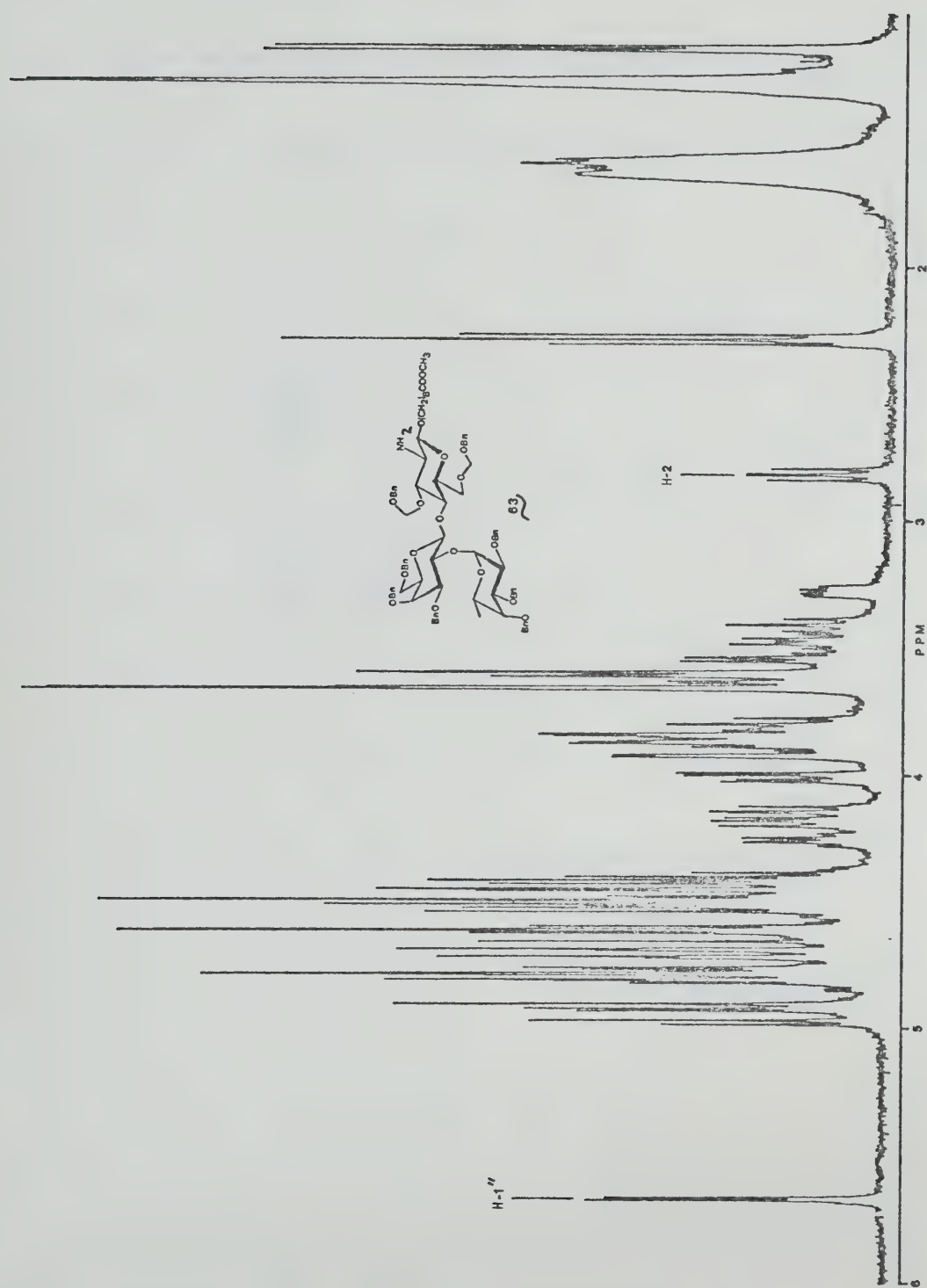


Fig. 13 The 400 MHz ^1H nmr spectrum of compound 63 in CDCl_3 .

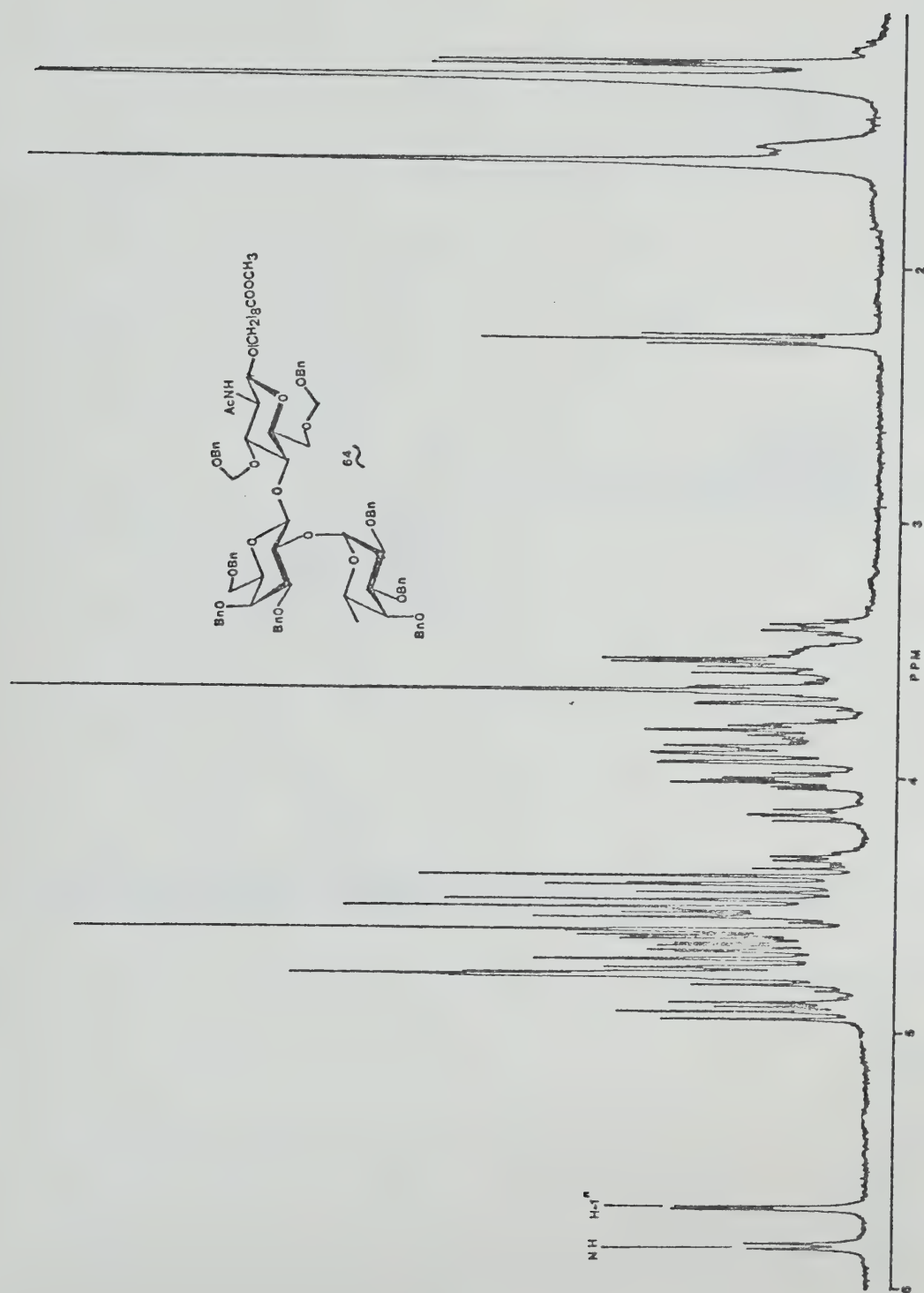


Fig. 14 The 400 MHz ^1H nmr spectrum of compound 64 in CDCl_3 .

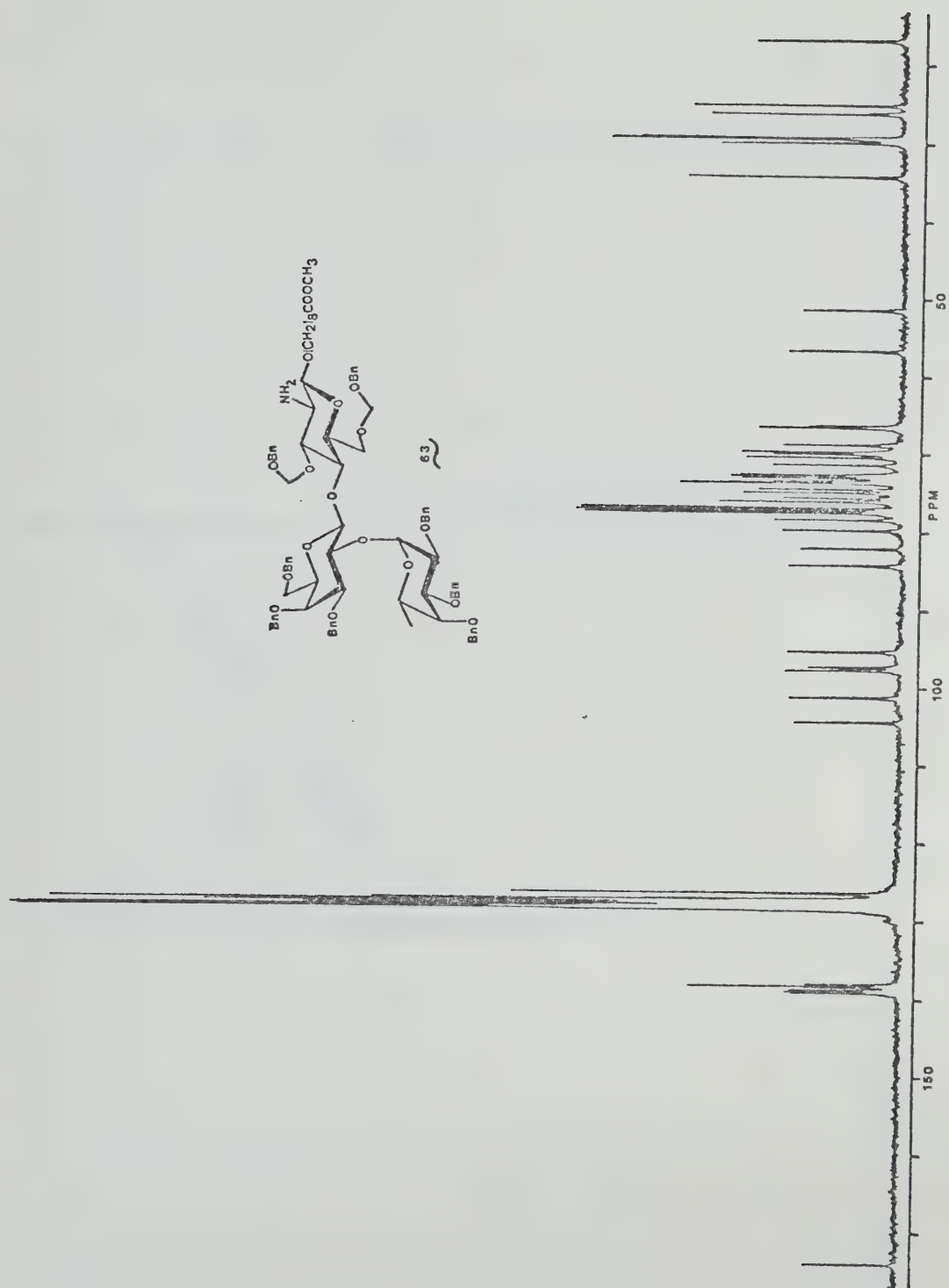


Fig. 15 The 100 MHz ^{13}C nmr spectrum of compound 63 in CDCl_3 .

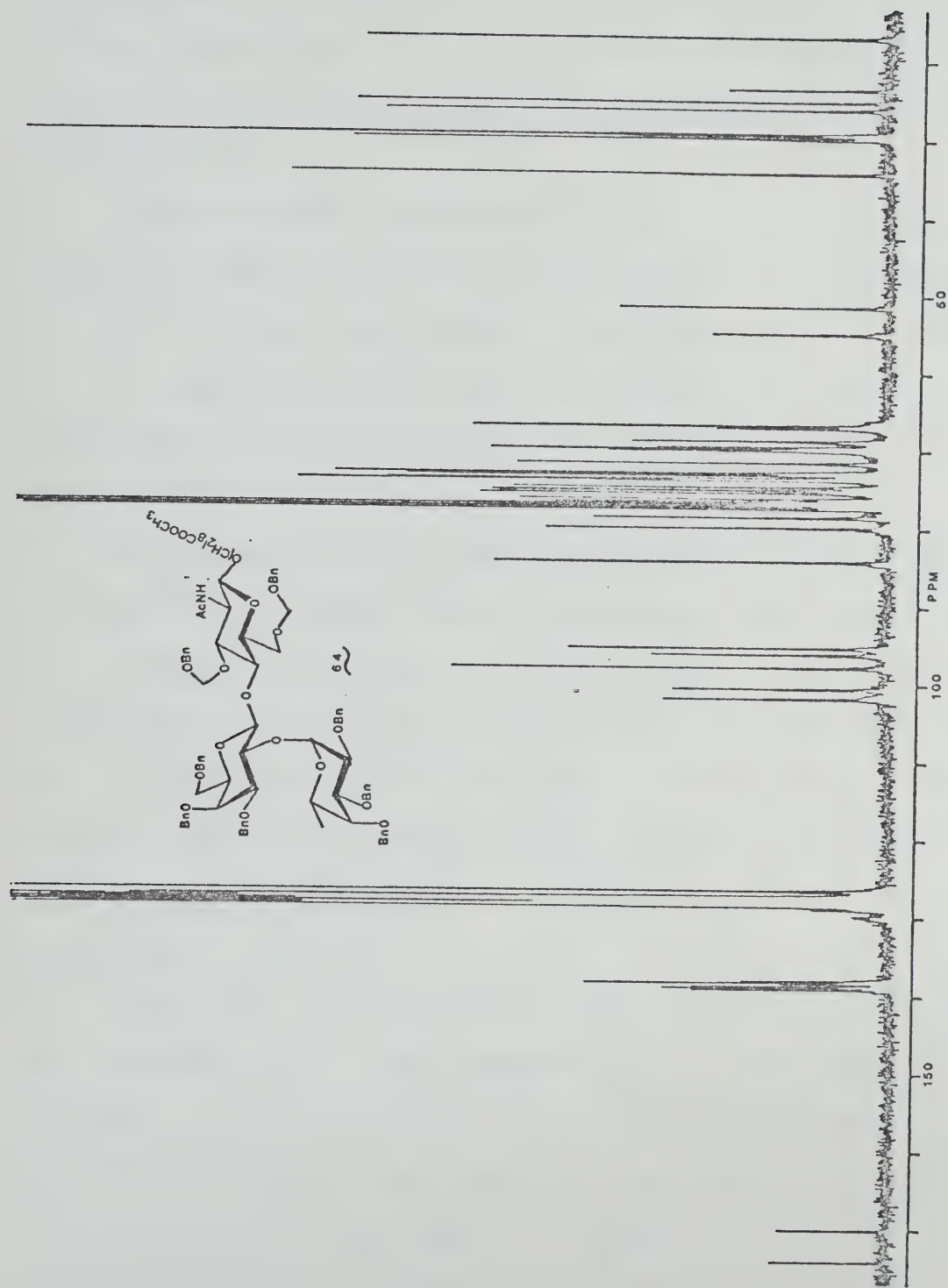


Fig. 16 The 100 MHz ^{13}C nmr spectrum of compound 64 in CDCl_3 .

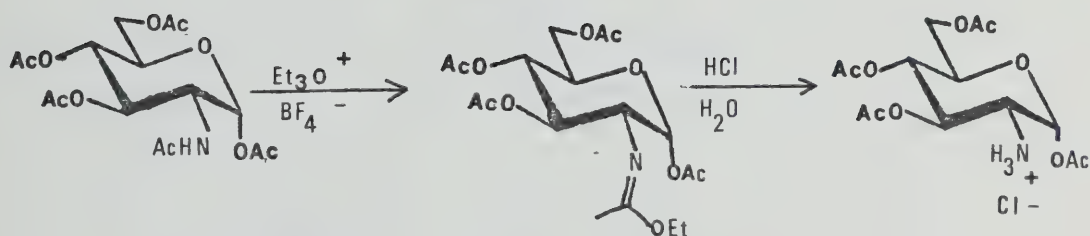
9.5 Hz) at δ 2.80 in the ^1H nmr spectrum of 63 resulted in the collapse of a doublet at δ 4.19 which would then be H-1. A large upfield shift of H-2 on N-deacetylation of 2-acetamido-2-deoxy-glycopyranosides has been documented in the literature.⁸⁸

The assignment of structure to 63 and 64 was thus relatively straightforward on the basis of a superficial analysis of their nmr spectra. Confirmation of the validity of these assignments was achieved by the N-acetylation of 63 (acetic anhydride/methanol) which resulted in its quantitative conversion to 64.

The N-deacetylation observed during the halide-ion catalyzed reaction is without precedent. The possibility exists that this may be, at least in some cases, a common side reaction during the α -glycosylation of acetamido sugars. If unnoticed, this may lead to significantly reduced yields, particularly when product isolation involves chromatography on an even slightly acidic support where the free amine may be trapped.

There can, in the author's view, be little doubt as to the mechanism of the N-deacetylation. The alkylation of amides by alkyl halides, in the presence of silver salts, is a well-established process for the preparation of O-alkyl imidates.⁹⁰ Hydrolysis of these imidates may be controlled so as to produce either the free amine

or to regenerate the amide.⁹⁰ This process has in fact found use in the area of carbohydrate chemistry where Hanessian applied the method⁹¹ for the N-deacetylation which involves formation of O-ethyl acetamidium tetrafluoroborates (Scheme 22).

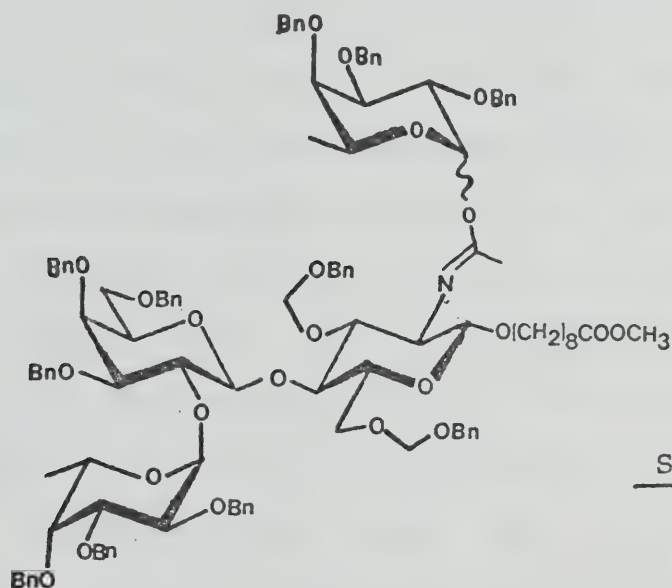


Scheme 22

The O-glycosylation of the amide function in acetamido sugars has also been found to occur as a side reaction during the Koenigs-Knorr reaction catalyzed by silver perchlorate.⁹² Several O-glycosyl disaccharide imidates have also been deliberately prepared.⁴⁵ The discovery of these imidates, in fact, formed the basis of a new method of glycosylation, the "imide procedure",^{42,43} which employs 1-O-imidyl- β -glycopyranoses in place of 1-halogeno sugars,

for the formation of α -linked glycosides. These anomeric imidates have been reported by Sinaÿ and co-workers⁴³ to be unstable to chromatography on silica gel.

It seems likely, then, in view of the large excess of fucosyl bromide used in the reaction with 62, that the major product (R_f 0.30) observed in the thin-layer chromatogram of the reaction mixture was the tetrasaccharide imidate shown in Scheme 23. The decomposition of this



Scheme 23

product in the course of the chromatography then produced 63 and 64 in a ratio of approximately 1:2. The isolation and proof of the intermediacy of this tetrasaccharide imidate was judged to be outside the scope of this research.

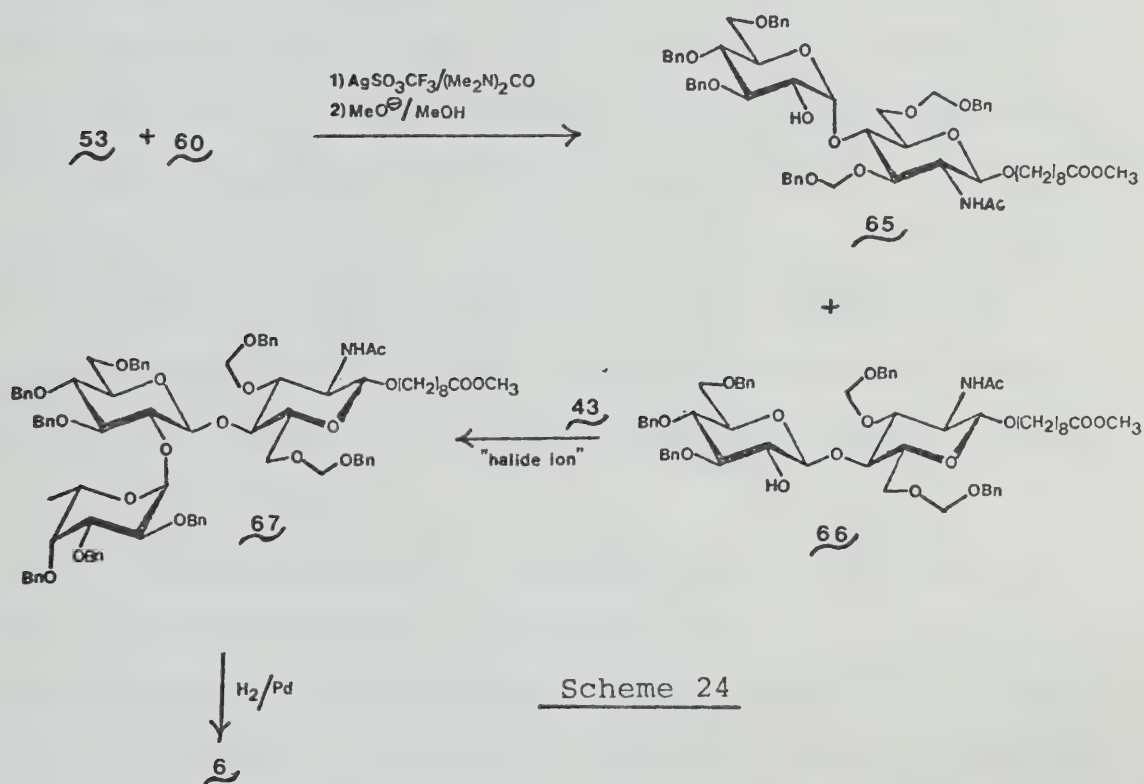
The hydrogenolysis of 64 proceeded smoothly and provided the H hapten (5) in 85% yield. The ^1H and ^{13}C nmr parameters for 5 are presented in Tables 1 and 2, respectively (Chapter 3). These parameters were in accord with the assigned structures.

4. 8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-4-O-[2-O-(α -L-fucopyranosyl)- β -D-glucopyranosyl]- β -D-glucopyranoside (6). The 4'-epi-H Hapten.

With supplies of both the glucosyl bromide 53 and the alcohol 60 available from the preceeding syntheses, the preparation of 6 required only three steps (Scheme 24). Koenigs-Knorr condensation of 53 and 60, under the conditions described by Hanessian and Banoub,⁸⁹ provided a mixture of disaccharide derivatives which could be separated by column chromatography after de-O-acetylation. The major product (Scheme 24) was the β -linked disaccharide 66 which was obtained in 31% yield. The β -configuration in 66 could be readily inferred from its partially relaxed (T_1) and decoupled ^1H nmr spectrum where the signal for H-1' appeared as a doublet, $J_{1',2'} = 8.5$ Hz at $\delta 4.72$. The α -linked disaccharide 65 (^1H nmr: H-1', $\delta 5.12$, $J_{1',2'} = 3.0$ Hz) was isolated in 11% yield. The α -L-fucosylation of 66 under halide-ion conditions provided the protected trisaccharide 67 in 57% yield. In this case, no evidence

for N-deacetylation was found. Hydrogenolysis of the protecting groups in 67 then produced the title trisaccharide 6 in 85% yield.

The ^1H and ^{13}C nmr spectral parameters are presented in Tables 1 and 2, respectively (Chapter 3) and are in accord with the assigned structure.



Scheme 24

CHAPTER III

THE CONFORMATIONS OF OLIGOSACCHARIDES
RELATED TO THE H AND LEWIS HUMAN BLOOD GROUPS

An appreciation of the conformational properties of the Le^a, Le^d and H haptens, and their 4'-epi-analogues, is basic to progress toward an understanding of their biological activities especially in terms of their interactions with receptor sites. Thus, for a meaningful analysis of the cross-reactivity of a pair of epimers to be presented, proof that these exist in near the same solution conformation must be put forward. Only after this can differences in specificity be interpreted uniquely in terms of the change in stereochemistry of a hydroxyl group and, consequently, the degree of involvement of that hydroxyl group in a receptor site.

The proposed solution conformations of the compounds under consideration were presented in Fig. 4. It must be emphasized that these two-dimensional illustrations should only be regarded as approximate representations of the true three-dimensional structures. Although an attempt was made to present these molecules from the angle which best illustrates their more important conformational aspects, space-filling models are required for a full appreciation of the finer points, particularly as regards overall topography.

Evidence, based in nmr spectroscopy, that these compounds reside in the conformations shown in Fig. 4 will be presented in this chapter.

Lemieux et al.⁷ have recently presented the results of a sophisticated conformational study of the A and B (Type 1) and Lewis oligosaccharides. Their conclusions regarding the preferred conformations of the Le^a and Le^d trisaccharides are of particular importance to this discussion. On the basis of modified hard-sphere calculations, which take into account the important contributions of the exo-anomeric effect (termed HSEA calculations),¹⁸ and the magnetic resonance properties of synthetic mono-, di, tri- and tetrasaccharide haptens, overwhelming support was provided that the Le^b tetrasaccharide adopts the conformation shown in Fig. 17a. Looking at this conformation from the right-hand side of the figure would provide the more familiar view presented in the simplified illustration of Fig. 17b.

Lemieux et al.⁷ were able to show that the Le^a and Le^d trisaccharides, which are partial structures of the Le^b tetrasaccharide, adopt essentially the same conformations as in their composite Le^b structure. Simplified illustrations of these conformations, and their relationship to the Le^b structure, are also presented in Fig. 17. These conformational similarities will result in similar localized

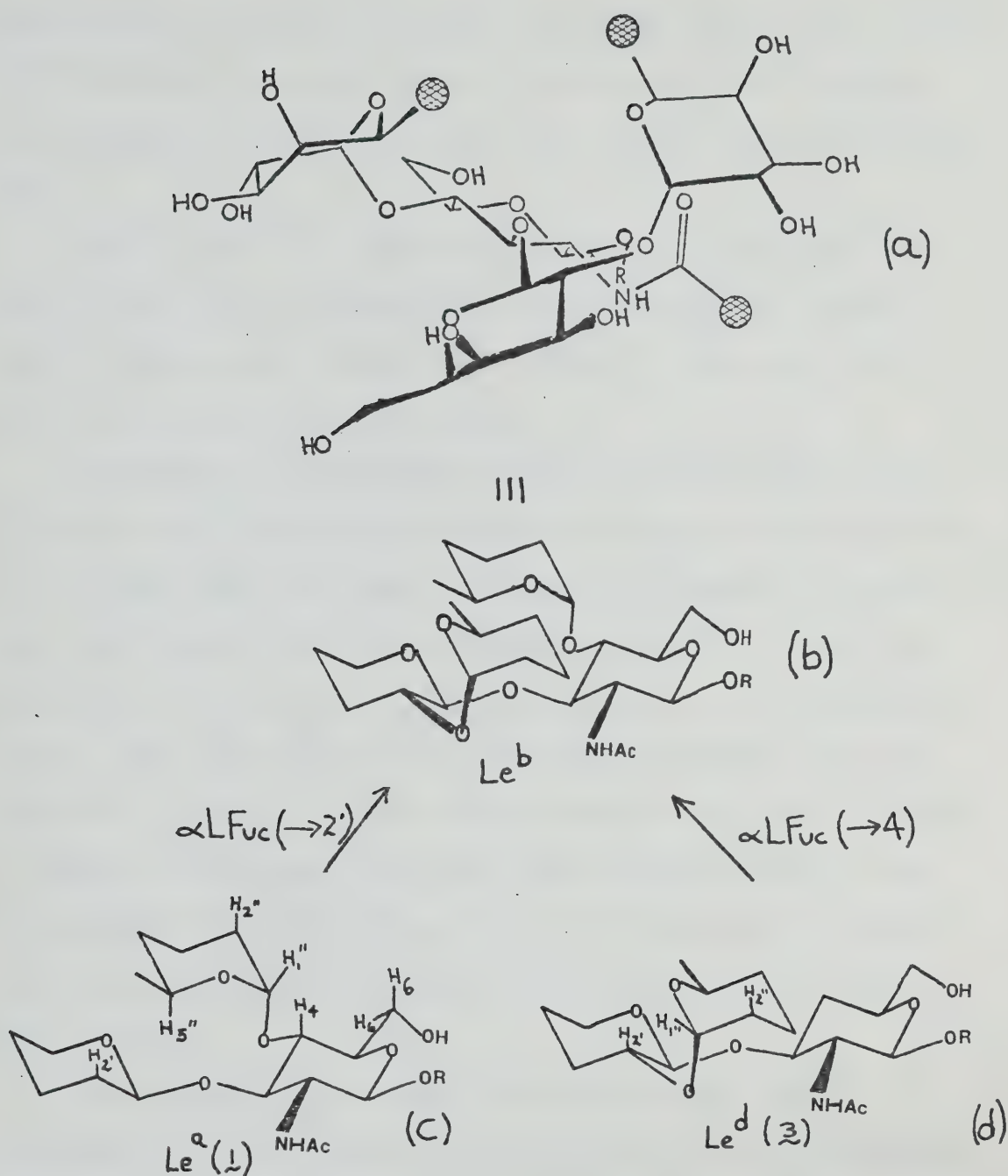


Fig. 17 The conformations of the Lewis determinants; a, the Le^b tetrasaccharide (from Lemieux et al.⁷); b, a different simplified view of the same structure; c, the Le^a trisaccharide; d, the Le^d trisaccharide.

topographies that explain well the observation¹² that anti-Le^b antibodies, raised against an artificial Le^b antigen, cross-reacted extensively with both the Le^a and Le^d structures.

The ¹H and ¹³Cnmr spectral parameters obtained⁷ for the Le^a and Le^d trisaccharides are presented in Tables 1 and 2, respectively, along with those for the new e-Le^a, e-Le^d, H and e-H trisaccharides.

In general, the conformations of the pyranose rings of sugar units in an oligosaccharide chain can be expected to be those preferred for the sugar as a simple glycoside; i.e., the βDGal, βDGlc and βDGlcNAc residues will be present in the ⁴C₁ and the αLFuc residue in the ¹C₄ conformations. The magnitudes of the coupling constants for the anomeric protons of these residues which are noted in Table 1 support these expectations. The conformations of the oligosaccharide chains are, therefore, expected to depend mainly on conformational preferences involving the glycosidic bonds.

The sensitivity of sugar-proton chemical shifts to differences in environment is well known and was elegantly demonstrated by Lemieux et al.⁷ in their comparisons of the δ-values of identical residues when present either as simple glycosides or as part of oligosaccharidic structures. Thus, for example, while H-1 of methyl α-L-fucopyranoside

TABLE 1. Comparisons of the ^1H nmr chemical shifts of compounds 1 - 6 in D_2O . Acetone (13) was used as internal standard at $\delta 2.48$.

Residue Position	8DC1cNAc						8DG1c						aLPuc							
	1	2	3	4	5	6	6'	1	2	3	4	5	6	6'	1	2	3	4	5	6
Le ^a (1) ^a	4.79	4.13	4.35	3.97	3.84	4.21	4.10	4.73	3.76	3.87	4.16	3.89	4.03	3.99	-	-	-	-	-	-
e-Le ^a (2) ^{b,c}	4.75	4.14	4.30	4.00	3.83	4.22	4.12	-	-	-	-	-	-	-	4.81	3.45	3.71	3.44	3.64	4.20
$\delta_1 - \delta_2$	0.04	-0.01	0.04	-0.03	0.01	-0.01	-0.02	-	-	-	-	-	-	-	-	-	-	-	-	-
Le ^d (3) ^a	4.65	4.07	4.29	3.76	3.77	4.18	4.01	4.87	3.85	4.11	4.15	3.95	4.04	4.00	-	-	-	-	-	-
e-Le ^d (4) ^{b,d}	4.65	4.04	-	3.74	3.71	4.19	4.02	-	-	-	-	-	-	-	4.94	3.66	3.94	3.66	3.71	4.19
$\delta_3 - \delta_4$	0.00	0.03	-	0.02	0.06	-0.01	-0.01	-	-	-	-	-	-	-	-	-	-	-	-	-
H (5) ^{b,e}	4.74	3.98	3.90	4.05	3.70	4.23	4.02	4.79	3.93	4.12	4.15	3.91	4.07	4.02	-	-	-	-	-	-
e-H (6) ^{b,f}	4.75	3.94	3.91	-	3.71	4.23	4.05	-	-	-	-	-	-	-	4.83	3.74	-	3.67	3.71	4.16
$\delta_5 - \delta_6$	-0.01	0.04	-0.01	-	-0.01	0.00	-0.03	-	-	-	-	-	-	-	-	-0.03	-0.01	-	-0.01	0.01

^a Values taken from Lemieux et al. at 270 MHz. ^b Determined by selective proton-decoupling, in conjunction with partially relaxed spectra, at 400 MHz. ^c $J_{1,2} = 8.8$ Hz, $J_{1',2'} = 7.9$ Hz, $J_{1'',2''} = 3.6$ Hz. ^d $J_{1,2} = 7.6$ Hz, $J_{1',2'} = 8.5$ Hz, $J_{1'',2''} = 4.0$ Hz. ^e $J_{1,2} = 8.2$ Hz, $J_{1',2'} = 7.5$ Hz, $J_{1'',2''} = 2.8$ Hz. ^f $J_{1,2} = 8$ Hz, $J_{1',2'} = 8$ Hz, $J_{1'',2''} = 3$ Hz.

provides its signal at 5.01 ppm, when the α -L-fucopyranosyl residue is present in the Le^a or Le^d structures this proton resonates at 5.27 and 5.44 ppm, respectively. Such significant changes in chemical shift are not unexpected since these three glycosides possess substantially different aglyconic structures which are necessarily in close proximity to the anomeric proton. Smaller chemical shift differences would naturally be expected as the protons become more removed from the aglyconic structure. This, however, need not be the case in an oligosaccharide where inter-residue interactions may occur. Thus, while H-5 of methyl α -L-fucopyranoside resonates at 4.27 ppm, in the Le^d structure this proton provides its signal at 4.56 ppm and in the Le^a structure at 5.10 ppm. A deshielding of 0.83 ppm, in the Le^a case, for a proton five bonds removed from the aglyconic carbon is truly remarkable. This downfield shift has been attributed¹⁰ to the electrostatic deshielding of H-5" by both O-3 and O-5', as indicated in Fig. 17c. Support for this conformation, based on specific nuclear Overhauser enhancements, will be presented later.

The sensitivity of the proton chemical shift to its environment is also clearly apparent by comparing, in Table 1, the δ -values of the protons of a given sugar residue when this residue is present in different oligosaccharidic structures. These chemical shifts should then serve as

TABLE 2. Comparisons of the $^{13}\text{Cnmr}$ chemical shifts of compounds $\underline{1} - \underline{6}$ in D_2O .
Dioxane (-2%) was used as internal standard at $\delta 67.40$.

Residue	SDGlcHAc						SDGal						SDGlc						aLFruc					
Position	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
$\text{Le}^{\text{a}} (\underline{1})^{\text{a,b}}$	101.80	56.72	77.06	73.42	76.36	60.80	103.66	71.46	73.34	69.25	75.51	62.52	-	-	-	-	-	-	98.92	68.76	70.09	72.87	67.67	16.26
$\text{e-Le}^{\text{a}} (\underline{2})^{\text{b,c}}$	101.79	56.61	77.33	73.63	76.83	60.58	-	-	-	-	-	-	103.20	73.95	76.21	71.37	76.20	62.62	99.13	68.63	70.03	72.84	67.39	16.36
$\delta_1 - \delta_2$	0.01	0.11	-0.27	-0.21	-0.47	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-0.21	0.13	0.06	0.03	0.28	-0.10
$\text{Le}^{\text{d}} (\underline{3})^{\text{a,b}}$	102.67	55.67	78.37	69.62	76.32	61.72	101.03	77.50	74.39	70.00	75.94	61.96	-	-	-	-	-	-	100.33	68.96	70.33	72.67	67.33	16.06
$\text{e-Le}^{\text{d}} (\underline{4})^{\text{c,d}}$	102.60	55.71	79.11	69.48	76.41	61.77	-	-	-	-	-	-	100.83	79.34	77.52	70.61	76.80	61.67	100.38	69.05	70.37	72.71	67.40	16.03
$\delta_3 - \delta_4$	0.07	-0.04	-0.74	0.14	-0.09	-0.05	-	-	-	-	-	-	-	-	-	-	-	-	-0.05	-0.09	-0.04	-0.04	-0.07	0.03
$\text{H} (\underline{5})^{\text{b,c}}$	101.89	56.25	73.12	77.26	76.12	61.13	101.28	77.36	74.43	69.98	76.02	61.86	-	-	-	-	-	-	100.28	69.14	70.55	72.56	67.73	16.11
$\text{e-H} (\underline{6})^{\text{c,d}}$	101.88	56.39	72.98	77.48	76.12	61.07	-	-	-	-	-	-	101.04	78.91	77.81	71.35	76.85	61.55	100.19	69.12	70.53	72.58	67.77	16.12
$\delta_5 - \delta_6$	0.01	-0.14	0.14	-0.22	0.00	0.06	-	-	-	-	-	-	-	-	-	-	-	-	0.09	0.02	0.02	-0.02	-0.04	-0.01

^a Values taken from Lemieux et al.⁷ at 67.89 MHz. ^b Assignments were made using selective proton irradiation.
^c Recorded at 100.6 MHz. ^d Tentative assignments.

sensitive indicators of the environments of their corresponding hydrogen atoms.

Comparison of the chemical shifts of the protons of the β DGlcNAC and α LFuc residues within the three pairs of epimers shows these to be in excellent agreement, the $(\delta-\delta)$ values being very near zero. This near-constancy of the chemical shifts of the protons of a common residue on going to its 4'-epimer is regarded (see the following discussion) as strong evidence that these residues remain in near-identical environments and, therefore, that the epimeric pairs of haptens reside in essentially identical conformations. The particularly good agreement of the chemical shifts for the α LFuc residue in the $\text{Le}^{\text{d}}/\text{e}-\text{Le}^{\text{d}}$ and $\text{H}/\text{e}-\text{H}$ pairs requires this residue to be substantially removed from the 4' position and this will be seen later to be the case. The excellent agreement of the ^{13}C chemical shifts for the fucosyl residue within these two pairs of epimers (Table 2) are in further support of this contention. Indeed, the $(\delta-\delta)$ values for these pairs are regarded as being within experimental error.

The sensitivity of ^{13}C chemical shifts to minor changes in hybridization is well known.⁹³ Differences in ^{13}C shifts of common units in different oligosaccharidic structures need not, therefore, indicate significant differences in overall conformation but may be due to minor changes in

valence angles, particularly about the glycosidic linkages.⁷ Nevertheless, the generally good agreement observed (Table 2) for the carbon resonances of units common to a pair of epimers is further support that all these pairs maintain essentially identical conformations. The difficulty in interpreting small carbon chemical shift differences in terms of conformational changes, for the reasons described above, puts the onus of establishing the absolute conformations of these structures back onto $^1\text{Hnmr}$ spectroscopy.

Evidence has so far been presented only that each pair of epimers resides in essentially the same conformation and should therefore present a similar topography, with the exception of the area immediately surrounding the 4'-position, to a receptor site. The establishment of the absolute conformations rests on the results of specific nuclear Overhauser enhancement (N.O.E.) studies.

Homonuclear $^1\text{H}\{-^1\text{H}\}$ N.O.E. studies have found wide application in structural stereochemical work.⁹⁴ The N.O.E. experiment consists of selectively irradiating the signal for one or more protons in the $^1\text{Hnmr}$ spectrum of a compound and observing the enhancements of the signals for other protons present in the same molecule. The r^{-6} dependence of this enhancement, where r is the distance between the irradiated and observed protons, makes such measurements highly sensitive to small changes in the separation of

these protons. When such protons are located on different sugar units of an oligosaccharidic structure even minor changes in conformations, which result in a change in their spatial separation, will therefore lead to a readily detectable difference in N.O.E.'s.

With the advent of Fourier-Transform high-field spectrometers, measurements of N.O.E.'s are made with confidence to within $\pm 2\%$.⁹⁵ Absolute N.O.E. effects are highly sensitive to a number of experimental factors including concentration, the presence of paramagnetic substances, the complexity and nature of the molecule under investigation, the strength of the magnetic field and the measurement itself. Therefore, the absolute value of a N.O.E. is not itself significant except in the sense that a strong observed N.O.E. requires the close proximity of the two hydrogen atoms. However, the relative values of N.O.E.'s for two or more hydrogens within the same molecule and obtained at the same time through the irradiation of a specific hydrogen are significant in the sense that the hydrogen which has the greatest N.O.E. must be closest to the hydrogen that was irradiated. Thus, although the absolute value is only of qualitative value, the ratios of N.O.E.'s measured at the same time are at least of semi-quantitative value.

The results of a typical N.O.E. experiment are presented in Fig. 18, where the effect of irradiating the anomeric proton of the α LFuc residue (H-1") in the e-Le^d trisaccharide (4) is shown. Such experiments could be conducted since the H-1" signals in all the haptens were well separated from the other signals in the spectrum and these equatorial hydrogens are essentially free of interactions with other intra-unit hydrogens except for the neighboring H-2" atoms. The N.O.E.'s can be most conveniently determined by recording, in alternating fashion, the normal and irradiated spectrum and then computer-subtracting one spectrum from the other.

In the spectrum where H-1" is being irradiated, and is therefore saturated, this proton will no longer provide a signal while the signals of protons proximate to H-1" will be enhanced. Consequently, subtraction of the irradiated spectrum from the normal spectrum (Fig. 18a) will provide a difference spectrum (Fig. 18b) where H-1" will appear as a negative signal of full intensity (i.e., this signal will integrate for one proton), the unenhanced signals will cancel out and the enhanced signals will appear as signals of fractional positive intensity. The N.O.E.'s of these latter signals may then be expressed as a percentage of the irradiated signal. The N.O.E.'s obtained in this manner, when irradiating H-1" in the six trisaccharides

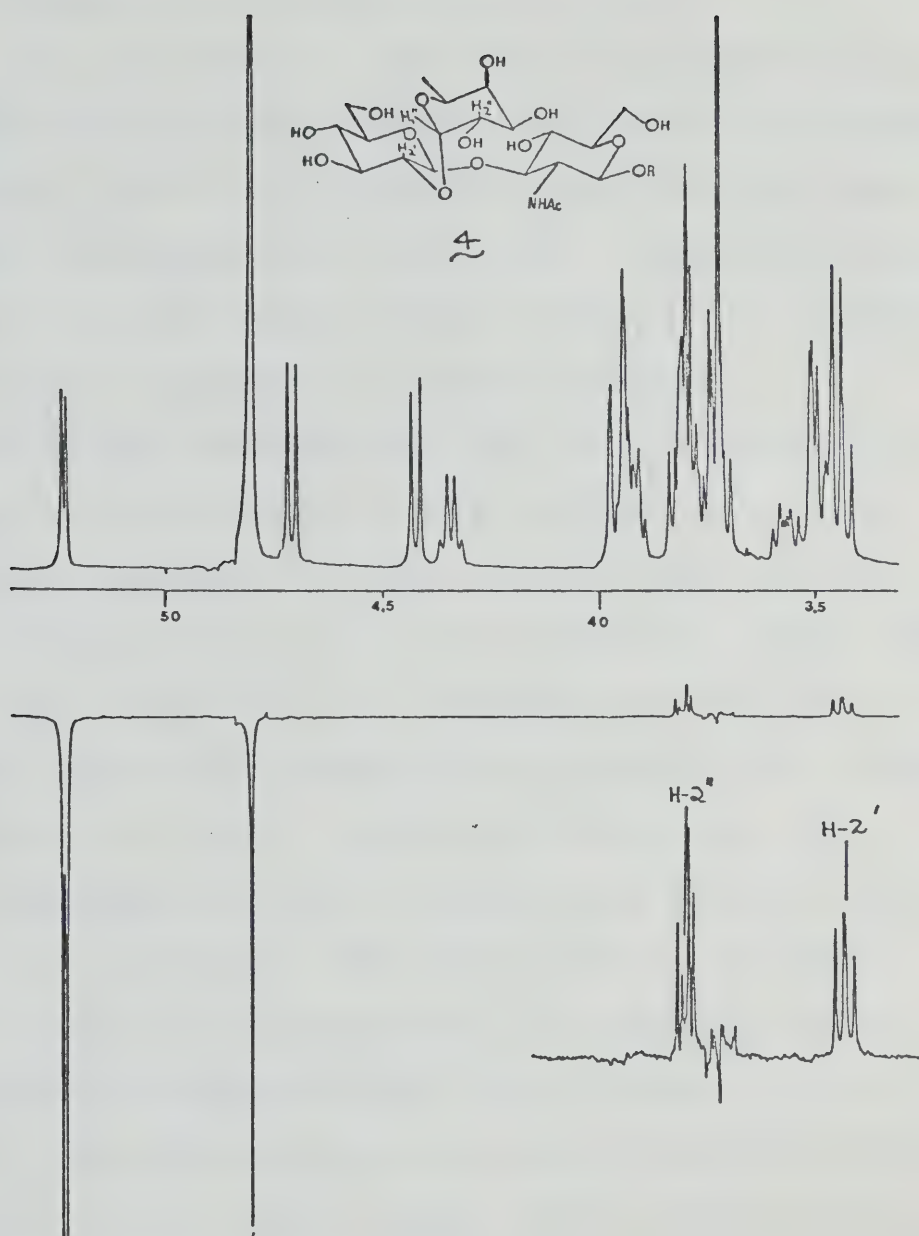


Fig. 18 The N.O.E. enhancements observed as a result of irradiating H-1'' in the 400 MHz spectrum of compound 4 in D₂O: a, the normal spectrum; b, the N.O.E. difference spectrum.

under consideration, are presented in Table 3.

As seen from Table 3, significant enhancements of the H-2" atoms were observed on irradiation of the neighboring H-1" atoms. This is in keeping with the fact that these two atoms are separated by only 2.36\AA .⁷ These enhancements therefore can serve as an internal standard for interpreting the N.O.E.'s observed for other hydrogens.

For the Le^a trisaccharide (Fig. 17c), the HSEA calculations of Lemieux et al. placed H-1" and H-4 at an internuclear distance of 2.68\AA in accord with relative N.O.E.'s observed for H-4 and H-2" (Table 3). These calculations also placed H-1" at a distance of 2.69\AA from C-6, a distance that would require strong interactions between H-1" and the H-6 atoms. Indeed, the observation of a readily measurable N.O.E. for these atoms (Table 3) supported this conclusion. The highly abnormal chemical shift of H-5" in 1 was attributed, as noted earlier, to the electrostatic deshielding of this hydrogen by both O-3 and O-5'. The HSEA calculations placed this proton directly above both of these oxygen atoms, and at a distance of only 2.40\AA from H-2'. Indeed, irradiation of H-5" caused an enhancement of 5% for its syn-clinal H-4", which was estimated to be 2.45\AA from H-5", and of 6% for H-2'.

Comparison of the N.O.E.'s observed when irradiating H-1" of the e-Le^a trisaccharide (2) with those reported

TABLE 3. Nuclear Overhauser Enhancements

Data observed when irradiating the anomeric proton of the LFuc residue (H-1") of compounds 1 - 6 in D₂O at 400 MHz.

Compound	N.O.E. (%)		
	H-2"	H-A ^a	Other
Le ^a (1)	12	8	H-6's (5 and 2)
e-Le ^a (2)	12	9	H-6's (5 and 1)
Le ^d (3)	14	16	---
e-Le ^d (4)	8	7	---
H (5)	6	7	---
e-H (6)	6	7	---

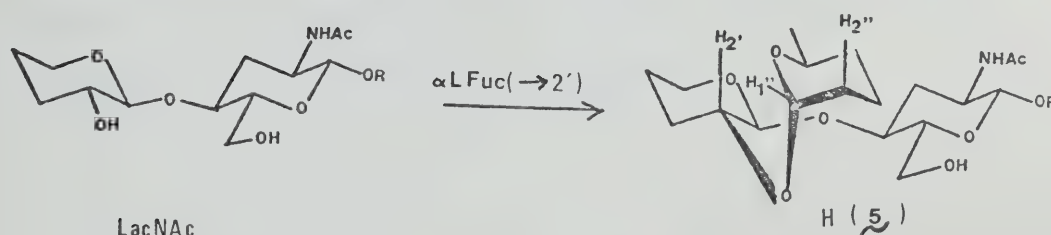
^a H-A refers to the proton at the aglyonic carbon; H-4 in 1 and 2, and H-2' in 3 - 6.

for the Le^a hapten (1) (Table 3) can leave no doubt that the αLFuc residue is oriented identically with respect to the $\beta\text{DGlcNAc}$ unit in both of these structures. In addition, the nearly identical chemical shift for the anomalous H-5'' in both 1 and 2 requires the same deshielding effects to be present and, consequently, requires the βDGlc unit in 2 to have near the same orientation as the βDGal unit 1.

Considerations similar to those described above allowed Lemieux et al.⁷ to show that $\alpha\text{-L-fucosylation}$ of the Le^c disaccharide [$\beta\text{DGal}(1\rightarrow3)\beta\text{DGlcNAc}$] to provide either the Le^a , Le^d or Le^b structures caused no appreciable change in the conformation of this disaccharide. This is the case since this conformation resides in a potential-energy well that is steep with respect to changes in angles about the βDGal glycosidic linkage as a result of both the *exo-anomeric* effect and inter-unit attractive and repulsive Van der Waals' forces.⁷ The Le^d trisaccharide (3) was shown to reside in a conformation which places the αLFuc unit to the front and somewhat on top of the $\beta\text{DGlcNAc}$ unit, as illustrated in Fig. 17d. This orientation places H-1'' 2.44\AA from H-2' and would require the observation of similar N.O.E.'s for both H-2' and H-2'' when irradiating H-1'' . As can be seen from Table 3, this was found to be the case for both the Le^d and e-Le^d compounds. As noted earlier, the remarkable consistency of the chemical shifts for the αLFuc protons in 3 and 4 (and also in 5 and 6) required that this residue be

well removed from the 4'-position. The proximity of the anisotropic carbonyl of the β DGlcNAc residue to the α LFuc unit in 3 (see Fig. 17a) would lead to the expectation that the chemical shifts of the protons in this latter unit should be particularly sensitive to small changes in the relative orientations of these two sugars. Since no significant differences in the chemical shifts of the protons of either residue were found between 3 and 4, these units are expected to be in the same relative orientations. The conformation of 4 would then be essentially identical to that proposed for 3.

The conformation of the β DGal(1 \rightarrow 4) β DGlcNAc disaccharide (N-acetyl-lactosamine, LacNAc) was shown⁷ to reside in the conformation illustrated in Scheme 25, and was found to be



Scheme 25

in a potential-energy well similar to that described for the Le^C disaccharide. There is thus no reason to expect that α -L-fucosylation of the 2'-hydroxyl group of LacNAc, to provide the H-structure, should appreciably alter this conformation. The orientation of the α LFuc unit relative to the β DGal unit in 3 and 5, and the β DGlc unit in 4 and 6, may, in addition, be expected to be the same.⁷ This contention is supported by the N.O.E.'s listed in Table 3 where, in each of these four compounds, the enhancements observed for H-2' and H-2'', while irradiating H-1'', were essentially identical.

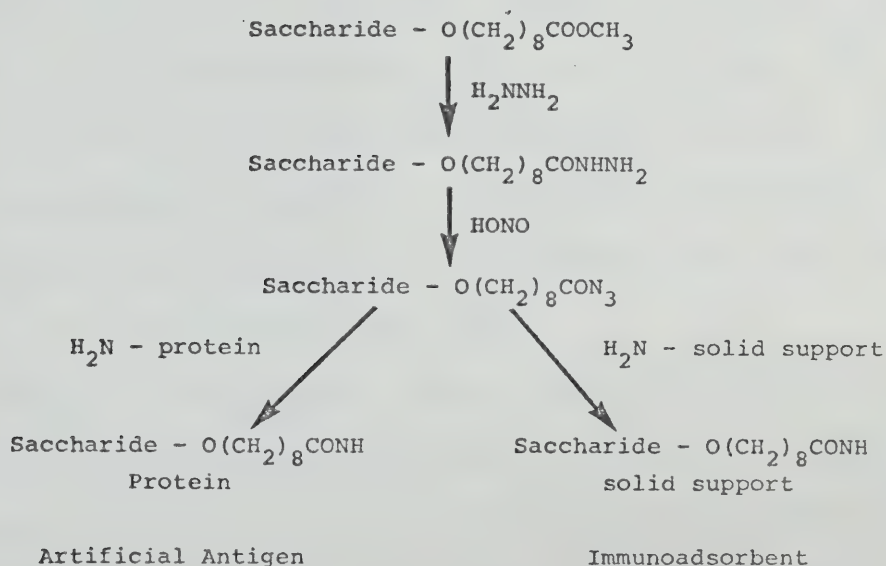
On the basis of the foregoing nmr evidence, it may then be expected that each of a pair of epimers possesses essentially the same conformational preferences for the glycosidic bonds. Differences in immunochemical specificities can then be assigned, with some confidence, to the degree of involvement of the area surrounding the 4'-position in a recognition phenomenon.

CHAPTER IV

IMMUNOCHEMISTRY

A. Preparation of Artificial Antigens and Immunoabsorbents; Rabbit Immunizations

The four trisaccharide haptens whose syntheses were described in Chapter II were prepared as their 8-methoxycarbonyloctyl glycosides¹⁰ in order to provide a linking-arm for the preparation of artificial antigens¹¹ and immunoabsorbents.¹² The general procedure is outlined in Scheme 26.



Scheme 26

This procedure involves the preparation of the hapten acyl-azides by way of the acyl-hydrazides. The acyl-hydrazides were prepared from the corresponding methyl esters by reaction with hydrazine-hydrate, either neat or in admixture with ethanol. The hydrazine-free hydrazides were obtained by evaporation of the reaction mixture under reduced pressure and filtration of the residue through a column of Biogel-P2. Nitrous acid oxidation then provided the acyl-azides for reaction either with an aminated solid support to produce the immunoadsorbent or with a carrier molecule to prepare the artificial antigen.

The solid support used in this work was a silylated Chromosorb P⁹⁶ which had an active surface amino-group incorporation of 3-6 $\mu\text{mole/g}$. After attachment of the hapten, at an incorporation of 0.4-0.5 $\mu\text{mole/g}$, the unreacted amino groups were acetylated so as not to confer any ion-exchange properties to the immunoadsorbent. An immunoadsorbent prepared in this manner can bind approximately 6 mg of antibody per gram.

Bovine serum albumin (BSA) was used as the carrier molecule: this protein possesses 57 free amino groups per molecule (molecular weight $\sim 65,000$)⁹⁷ and has been widely used for the preparation of so-called "artificial" antigens. An incorporation of 14-20 haptens per BSA molecule was achieved. As estimation of the degree of hapten

incorporation into both the artificial antigen and the immunoadsorbent was obtained by sugar analysis using the phenol-sulfuric acid method.⁹⁸ The details are provided in the experimental section.

The Le^a , e-Le^a , Le^d , e-Le^d , H and e-H antigens were each administered to San Juan rabbits, in groups of 3, in Freund's complete adjuvant. The multiple-boost immunization schedule used was that described by Lemieux, Bundle and Baker¹¹ as Protocol A. The rabbits were exsanguinated after four weeks. After clot removal, the individual sera were stabilized with sodium azide (0.5%) and stored at 0-4°C.

B. Characterization of Anti-Sera

The general success of these immunizations was evident from immunodiffusion assays. All sera gave lines of precipitation against the corresponding immunizing antigen on immunodiffusion analysis⁹⁹ using agarose gel containing 0.1% BSA. All the pre-immune sera were negative. Considerable variations were observed in the intensity of these lines, even within the sera obtained from rabbits immunized with the same antigen. All the anti- Le^a sera gave strong precipitin lines against the e-Le^a antigen and vice-versa. The anti- Le^d and anti-H sera generally gave weak precipitin lines against their corresponding immunizing antigens and

slightly weaker lines still against the 4'-epimeric antigens. The anti-e-Le^d and anti-e-H sera, on the other hand, all gave extremely strong lines against their immunizing antigens and generally very weak lines against the 4'-epimeric analogues.

In order to attempt to quantitate these observed cross-reactions, a method that would detect only the antibodies directed against the carbohydrate-portion of the antigen was required. Quantitative precipitin assays¹⁰⁰ are normally used in order to determine the antigen-specific titers of immune-sera but this method would also detect antibodies directed against the protein portion common to all the immunizing hapten-BSA antigens. As will be seen later, a large proportion of the antibodies raised against these synthetic antigens are in fact directed towards the BSA structure.

A new and exceedingly simple method for determining the titers of hapten-specific antibodies in immune-sera was developed in these laboratories by J. LePend¹⁰¹. This method, based on the availability of the haptenated immuno-adsorbents, has been termed 'quantitative batch immuno-adsorption' (QBIA) and is essentially an adaptation of the 'quantitative micro-method for measuring antibody' described by Gill and Bernard.¹⁰² The QBIA experiment consists of agitating a 1 mL solution of serum, of known concentration,

in phosphate-buffered saline (PBS), with an immunoadsorbent (IA) until antibody adsorption is complete (ca. 2.5 h). After centrifugation, the supernatant, containing unbound protein, is removed and the IA is washed with PBS until the washes are free of U.V. (280 nm) absorption. This procedure leaves bound to the IA only those antibodies that have a sufficiently high affinity for its hapten. Addition of saline 2% ammonium hydroxide (1 mL) to the dried IA then effects the denaturation and concomitant desorption of these antibodies. The optical density of the supernatant solution, read against the corresponding solution obtained by identical treatment of an unhaptenated IA, then allows the calculation of the antibody concentration using an extinction co-efficient of $E_{280}^{1\%} = 14$ for immunoglobulin G (IgG),¹⁰¹ the major immunoglobulin to be synthesized during the secondary immune response.¹⁰³

The results of a series of such experiments are shown in Fig. 19 where the anti-serum obtained from a rabbit immunized with the Le^a -BSA antigen was adsorbed, at several dilutions, with 30 mg of Le^a -IA. The adsorption curve is reminiscent of a Langmuir adsorption isotherm¹⁰⁴ where, clearly, the IA became saturated with antibody when the serum concentration was near 150 μ L/mL in PBS. The linearity of the curve at low serum concentration requires that, in this region, essentially all the anti- Le^a antibodies are

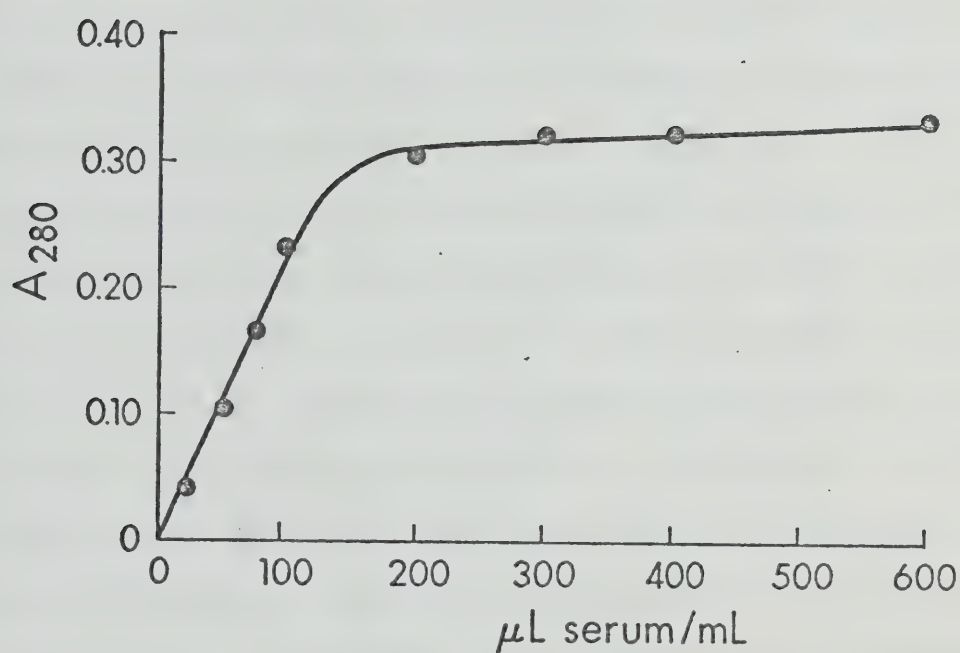


Fig. 19 QBIA assays of serum 2 (anti-Le^a-BSA). The absorbance of the 2% ammonium hydroxide solution, containing the antibodies desorbed from a constant weight of the Le^a-IA (30 mg), is plotted against the concentration of the adsorbed serum.

bound until the saturation level is achieved. The quantity of high affinity hapten-specific antibody present in small volumes of serum can thus be determined.

The series of QBIA experiments described above is somewhat demanding in valuable serum and provides little information in the high serum-concentration range. The procedure for determining the antibody-titers against a large number of different IA's has therefore, in practice, consisted of single point determinations at one concentration (usually 100 μ L/mL) which was verified to be well below the concentration required to effect saturation of the IA.

The antibody-titers of the anti-Le^a and anti-e-Le^a sera against both the immunizing antigen, obtained by the quantitative precipitin reaction in 2-4% polyethylene glycol,^{101,105} and its corresponding hapten, obtained by the QBIA method, are presented in Table 4. Comparison of these two values shows that only near one-third of the immune response is directed toward the carbohydrate structure of the BSA-antigens. The QBIA titers against the 4'-epimeric trisaccharide of the immunizing antigen and the two disaccharide partial structures of the Le^a hapten, β DGal(1 \rightarrow 3)- β DGlcNAc (Le^C) and α LFuc(1 \rightarrow 4) β DGlcNAc (Lewis-disaccharide, Le-disac), are also reported in Table 4.

TABLE 4. Characterization of the antibodies raised in rabbits against the Le^a and e-Le^a BSA antigens.

	Analysis of the serum (mg. antibody/mL)		% Cross-reaction with related structures		
Serum	Precipitin assay ^a	QBIA assay ^b	QBIA assay ^c		
			4'-epi	Le ^c	Le-disac
Immunization with the Le ^a BSA antigen					
1	4.33	1.17	76	22	32
2	4.98	1.64	98	56	35
3	8.66	2.30	89	36	54
Immunization with the e-Le ^a BSA antigen					
4	9.65	2.32	57	10	27
5	6.55	1.83	55	19	31
6	3.05	0.83	39	0	6

^aAgainst the immunizing antigen using the 2-4% polyethyleneglycol method.^{101,105}

^bAgainst the trisaccharidic hapten of the immunizing antigen.

^cAgainst the haptenated immunoabsorbants. These titers are expressed as a percentage of the total immunizing hapten titer (b).

As can be seen in Table 4, the anti-Le^a antibodies cross-reacted to a high degree with the e-Le^a structure. Indeed, the antibodies in the anti-Le^a sera 2 and 3 essentially all bound this epimeric hapten. This result would be explained if these antibodies were directed largely against the Le-disac portion common to both structures. The Le-disac structure, however, binds, on the average, less than half the antibodies that cross-react with the e-Le^a structure (Table 4). In the case of serum 2, where the Le^a/e-Le^a cross-reactivity is complete, only about a third of the antibodies bind this common unit. In fact, the cross-reactivity of the anti-Le^a antibodies with the Le-disac structure is seen, from Table 4, to be of the same extent as with the Le^c structure.

It could be expected that the anti-Le^a antibodies that possess combining-sites which bind substantial portions of the three sugar units could bind with disaccharide units of the Le^a determinant since the smaller structures could, in principle, occupy the binding site. Indeed, in the case of serum 2, as seen in Table 4, the Le^c and Le-disac IA's combined bind almost as much antibody (91%) as does the Le^a-IA. When this serum was adsorbed successively with excess amounts of these two IA's, however, only 67% of the anti-Le^a antibodies were removed. Isolation of the remaining activity on a column of Le^a-IA led to the

recovery of 33% of the total antibody.

The antibodies of serological interest would, of course, be those that require such a large portion of the surface of the Le^a determinant for binding that the incomplete disaccharide structures would bind only very weakly, if at all, with these antibodies. These antibodies, the latter 33%, would be candidates in this regard. It may be noted that, for this particular serum (serum 2), these antibodies, as was expected, reacted completely with the e-Le^a structure in the QBIA assay. Thus, while portions of all three sugar units are required for useful binding by these antibodies, the area surrounding the 4'-position of the β Gal residue is not likely involved in the binding.

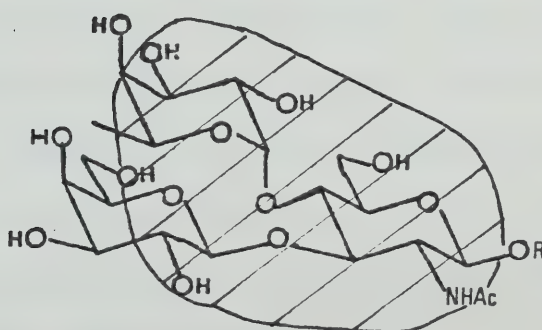
As noted earlier, the synthetic antigens (and IA's) possess the carbonyloctyl linking-arm which is, of course, not present in the natural antigens. Involvement of this linking-arm in the immune response has previously been demonstrated by Lemieux et al.¹¹ The possibility, then, existed that a high proportion of the antibody population produced against the synthetic Le^a-BSA antigen was, in fact, directed against the "reducing" end of the hapten including this linking arm. The observed cross-reactivities with the e-Le^a and partial disaccharidic structures might then result from the antibodies deriving a significant

binding energy from the β DGlcNAc-O(CH₂)₈ unit which is common to all the haptenated IA's.

Since the rabbit anti-Le^a-BSA sera obtained from these rabbit immunizations were found to be non-agglutinating, the anti-serum from a goat⁹⁶ that was previously immunized with the Le^a-BSA antigen was chosen to test the above proposal. The anti-Le^a activity of this goat serum was isolated on a column of Le^a-IA and, after desorption and neutralization, obtained as an antibody solution which strongly agglutinated OLe^{a+} human red-cells. The antibodies responsible for this agglutination of the natural antigens cannot be directed against the linking-arm since it is not present in the natural antigens on the red-cells. Passage of this antibody solution through a column of e-Le^a-IA removed 63% of the antibodies. The eluate, containing those species (37%) which do not bind the e-Le^a structure, still gave a strong precipitin line against Le^a-BSA in immunodiffusion assays but was virtually devoid of agglutinating activity against the same OLe^{a+} cells. Desorption of the antibodies from the e-Le^a column gave a solution which, after dialysis and appropriate concentration, contained only half the protein concentration of the unfractionated antibody solution yet still had essentially the same high agglutination titer. Since

these antibodies agglutinate the natural Le^a antigens, they cannot be expected to be strongly directed against either the linking-arm of the artificial antigen or the area immediately surrounding the 4'-position of the βGal residue in the Le^a determinant.

A consideration of the above discussion, coupled with the observations of Lemieux et al.¹² that anti- Le^a -BSA antibodies raised in rabbits cross-reacted very poorly (~10%) with the Le^b structure, allows some conclusions to be made regarding the specificity, in terms of structural requirements, of the anti- Le^a antibodies raised through this procedure. This specificity, as was shown is not necessarily unique to the Le^a determinant and the antibodies may be fooled. An attempt to illustrate the surface



Scheme 27

of the Le^a trisaccharide which is therefore believed to give rise to the Le^a specificity of the antibodies under consideration is presented in Scheme 27.

The anti-e-Le^a sera had titers against both the immunizing antigen and its trisaccharidic hapten of the same order as those found for the anti-Le^a sera (Table 4). The cross-reactivity of the anti-e-Le^a antibodies with the epimeric structure was, however, only near 50%, as determined by QBIA assays. This situation is distinct from the anti-Le^a sera and requires a substantial population of these antibodies to be directed toward the β DGlc residue. The very low cross-reactivity observed with the Le^c structure is in support of this conclusion.

The cross-reactivity of these anti-e-Le^a antibodies with the Le-disac structure is seen from Table 4 to be only moderate. This antibody population might be expected to bind both the Le^a and, of course, the e-Le^a structure (since the antibodies were raised against this structure) since these have the Le-disac residues in common. These antibodies were isolated on a Le-disac column and found, indeed, to cross-react completely with both trisaccharide haptens by the QBIA assay.

Removal of the Le^c and Le-disac activity from serum 4, by passage through columns of the respective IA's,

followed by isolation of the remaining anti-Le^a activity on a column of Le^a-IA gave a 20% yield of the total anti-e-Le^a antibody. This antibody population may be expected to have essentially the same specificity requirements (illustrated in Scheme 27) as that isolated from the anti-Le^a serum 2, in 33% yield, after identical treatment. That is, the antibodies would be expected to bind the Le^a determinant of the natural antigens. The results of tissue-immunofluorescence-staining experiments are unfortunately not available at this time to verify this expectation.

The characteristics of the antibodies raised against the Le^d, e-Le^d, H and e-H antigens will be discussed together for reasons that will soon become apparent. The titers of these sera against the immunizing antigens, the corresponding haptens and their 4'-epimeric analogues, and related structures are presented in Table 5. The titers of these sera against the immunizing antigens, again obtained by quantitative precipitin assays, are generally of the same order, except for serum 11, as those found previously. Rabbit 11 evidently possessed a very well-developed immune system.

While the quantity of antibody produced against the immunizing antigens is in the expected range for these

TABLE 5. Characterization of the antibodies raised in rabbits against the Le^d, e-Le^d, H and e-H BSA antigens.

Analysis of the Serum (mg. antibody/mL)			% Cross-reaction with related structures				
Precipitin Assay ^a	QBIA Assay ^b	QBIA Assay ^c					
		4'-epi	H-disac	Le ^c	H	e-H	
Immunization with the Le ^d BSA antigen							
7	5.25	0.75	72	0	10	0	-
8	2.56	0.71	67	17	39	0	-
9	6.84	1.31	60	0	47	0	-
Immunization with the e-Le ^d BSA antigen							
10	5.41	3.38	6	10	2	-	48
11	16.4	3.90	23	9	7	-	47
12	7.75	3.76	10	3	3	-	34
Immunization with the H BSA antigen							
					LacNac	Le ^d	e-Le ^d
13	4.33	1.17	73	0	57	0	-
14	4.33	0.94	78	0	36	0	-
15	3.53	0.92	83	0	30	0	-
Immunization with the e-H BSA antigen							
16	8.55	2.68	19	0	10	-	25
17	3.36	3.02	30	19	6	-	41
18	6.12	3.22	25	8	6	-	43

^aAgainst the immunizing antigen using the 2-4% polyethyleneglycol method.^{101,105}

^bAgainst the trisaccharidic hapten of the immunizing antigen.

^cAgainst the haptenated immunoadsorbents. These titers are expressed as a percentage of the immunizing hapten titer (b).

sera, glaring differences in the levels of hapten-specific antibody are apparent between the sera obtained by immunization with the antigens derived from the 'natural' structures (Le^{d} and H) and the 'unnatural' structures (e-Le^{d} and e-H).

The results listed in Table 5 can be appreciated on the basis that the rabbits used in these immunization experiments possess H determinants.^{106,107} Thus, the $\alpha\text{LFuc}(1\rightarrow2)\beta\text{DGal}$ (H-disac) portion of the H (Type 1) or Le^{d} and H (Type 2) determinants would be 'self' structures and, therefore, not prone for participation in the immune response. Evidently, to do otherwise would lead to anti-self antibodies and serious disease. In fact, the rabbits survived the immunizations in good health. Therefore, one may have predicted that the antibodies raised against the Le^{d} and H-BSA antigens would not cross react appreciably with the H-disac structure or the alternate trisaccharide determinant with which it has only the H-disac structure in common. The data in Table 5 clearly show that this was indeed the case. In fact, it is seen that the immune response avoided raising antibodies against these oligosaccharides compared to when the structure was 'non-self' as seen in the immunizations with the e-Le^{d} and e-H antigens. In these latter cases, the response against

the trisaccharide structures are seen (Table 5) to be near 3 times higher. Furthermore, an important fraction (~40%) of these antibodies were found to cross-react with the alternate epi-structure and would therefore appear to be directed against the α LFuc(1 \rightarrow 2) β DGlcNAc (e-H disac) unit.

As may be expected from these results, the antibodies raised against the Le^d or H-BSA antigens cross-reacted extensively (60-83%) with the epi form of the antigen. Thus, although the immune response attempted to avoid the trisaccharide, when it did become involved, the involvement was with those portions of the Le^d and H determinants that do not include the H-disac structure: the "reducing" ends of the haptens.

These results appear to confirm the expectation^{106,107} that rabbits possess H-determinants as self-determinants. Unfortunately, it has not been possible to date to have rabbit tissues or erythrocytes typed in this regard.

C. The Lewis Antigens and Secretor Status¹⁰⁸

The ABH (commonly referred to as ABO) and the Lewis human blood groups are of major importance to tissue transplantation including blood transfusion. Except for a rare (<0.1%) group of people known as Bombay types, A and/or B and/or H determinants occur on the endothelial cells of people.¹⁰⁹ These activities are also found in the

secretions of about 80% of people. The near 20% of individuals that do not secrete ABH active substances are termed non-secretors¹¹⁰ and are said to lack the secretor gene.¹¹¹ This matter became of interest as a result of the study of the distribution of the Lewis antigens in epithelial cells.¹² The Lewis antigens are also found on red cells but are transported in the plasma to this endothelial tissue from the epithelial cells where these are synthesized.¹¹² The results of this investigation are illustrated by the data in Table 6. These data were acquired employing highly specific anti-Lewis antibodies which were produced by way of artificial antigens and immunoadsorbents following procedures which were outlined earlier in this chapter. The tissues of the stomachs of a large number of healthy H blood group persons were examined using the technique of immunofluorescence staining.¹¹³ Tests were made for three of the four different Lewis determinants; namely, the Lewis-a, Lewis-b and Lewis-d antigens.

As seen from Table 6, there appeared to be a correlation between the persons who possess either the Le^b or Le^d determinants and the number of people who are secretors of ABH active substances. It is seen that no correlation at all exists between secretor status and the ABH antigens of the red cells. It should be recalled at this point that the Le^d determinant has been referred to as the H (Type 1)

TABLE 6. A comparison of statistics for Caucasian individuals that secrete ABH active substances and the statistics for the occurrence of Lewis antigens in these persons. The statistics are presented as approximate since these vary considerably with ethnic background.

Occurrence of Lewis Antigens				
<u>Secretors</u>	<u>Le^{a-d-}</u>	<u>Le^{a+d-}</u>	<u>Le^{a+d+} (Le^b)</u>	<u>Le^{a-d+}</u>
~80%	~1%	~20%	~70%	~10%
			~80%	

Occurrence of ABH Antigens			
<u>A</u>	<u>B</u>	<u>AB</u>	<u>H</u>
~42%	~8%	~3%	~47%

determinant and is the precursor to the Le^b determinant¹¹⁴ and that it is well established⁶ that the Le^d determinant can serve as the substrate for the development of so-called A (Type 1) and B (Type 1) antigens in individuals who have appropriately localized DGalNAc and DGal transferases; namely, A and B human blood group individuals, respectively. Thus, it could be presumed that the A or B antigens that are present in the secretions are of the Type 1 variety - and, therefore, their presence in the secretion, like the Le^b determinant, was related to the formation in the individuals epithelial cells of the precursor Le^d antigen. In other words, it seemed rational to hypothesize that ABH secretor status was dependent on the synthesis of the Le^d determinant in the epithelial cells. However, alas, the known forms for the Lewis-a, b and d determinants do not bind the lectin Ulex europaeus which is the reagent that is employed to test for H activity in secretions. This test is normally made by examining whether or not the saliva of the patient inhibits the agglutination of H red blood cells by the Ulex lectin. The red cells of these individuals are known to possess the H antigenic determinant which is strongly bound by Ulex europaeus.

The structural relationships between the Type 1 and Type 2 ABH human-blood group determinants are shown in

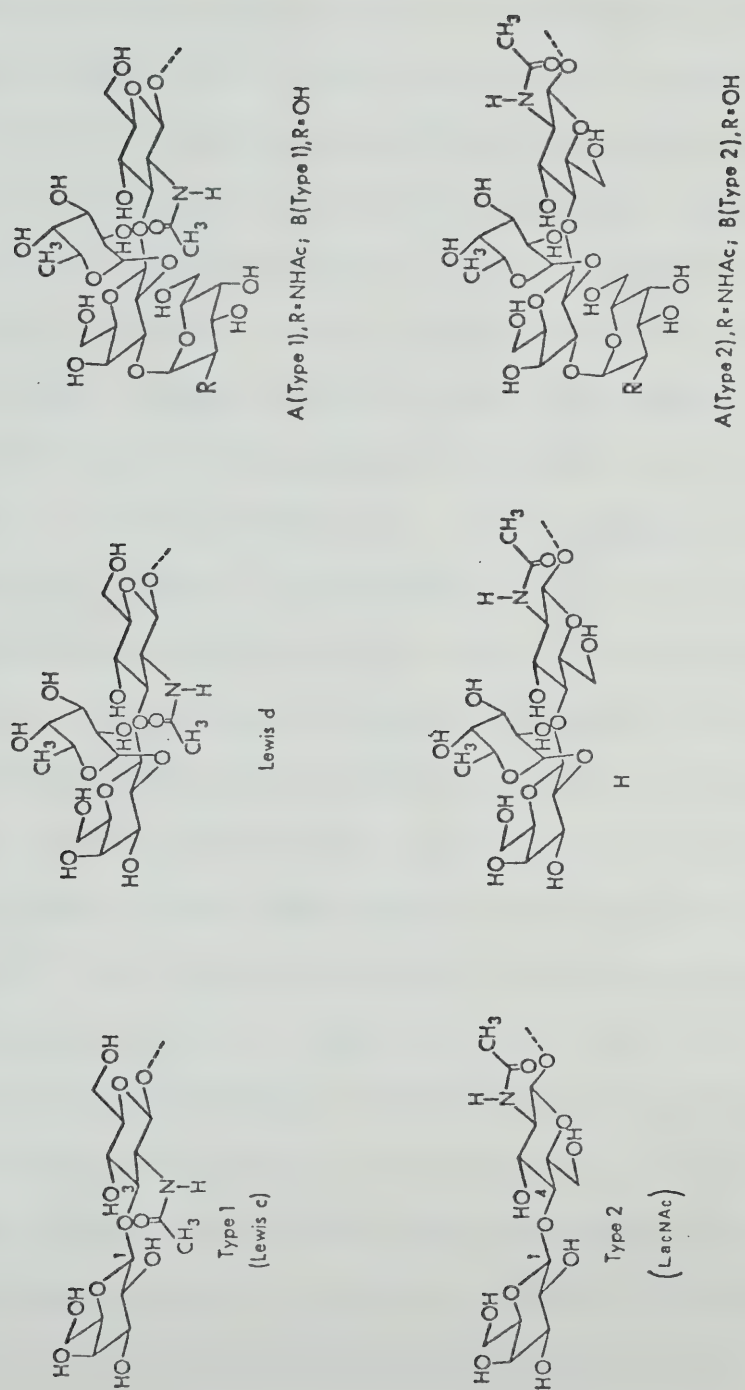


Fig. 20 Projection formulas to illustrate the preferred conformations for the Type 1 and Type 2 human blood group determinants.

Fig. 20. It is seen that, at the various levels of development, these have the same sugar composition. The differences arise because the precursor disaccharide for the Le^d determinant has a β DGal unit linked to the 3-position of a β DGlcNAc residue whereas in the Type 2 determinants this linkage is to the 4-position of the β DGlcNAc residue of the developing oligosaccharide. It was pointed out by Lemieux and co-workers⁷ that it would be truly extraordinary should the same enzyme system convert the Type 1 (Le^c) structure to the Le^d determinant and the Type 2 (LacNAc) structure to the H determinant. On the other hand, these conformational studies⁷ suggested that the 3'-hydroxyl group of the β DGal units of both the Le^d and H determinants are likely in stereochemically very similar environments and both these structures can be expected to be transformed to A and B determinants by the same enzyme systems.

Using the refined anti-Lewis reagents, Drs. Weinstein and Switzer¹² were able to clearly establish the presence of the Lewis antigens in normal stomach tissues of H blood group individuals according to the expectation based on the typing of their red cells. The discovery of the Le^d antigen on the tissues of Lewis-a and b negative persons except for one patient proved of interest since this Lewis-a, b and d negative person proved to be a nonsecretor. The

inference then was that all but a very small proportion of people possess either Le^a , Le^b or the Le^d antigens, most likely as glycoproteins on their epithelial cells.

It is considered of interest to speculate that the Le^a determinants of these antigens have the glucosamine residue in the acetylated form since whether or not this residue is in the amine or acetamido form might not be expected to influence the α -L-fucosylation at the remote 4-position. Since the antibodies used to locate the Lewis antigens were raised in rabbits against the β DGal(1 \rightarrow 3)[α LFuc(1 \rightarrow 4)] β DGlcNAc-BSA antigen, it seems probable, recalling Scheme 27, that the natural antigen also contained the β DGlcNAc residue since the antibodies are expected to bind in the region near the acetamido group. This is likely not the case for the antibodies directed against the Le^b and Le^d determinants since it is expected that these reagents are binding these antigenic determinants at regions remote from the acetamido (or amino, see below) group.¹⁰⁸ This matter is pointed out since it was instrumental to the formulation of an interesting question; namely, is it possible that a person is Le^a not because the person is deficient in the α LFuc transferase required to fucosylate the Type 1 precursor to the Le^d structure but because, instead, the Type 1 disaccharide unit in the N-acetylated form is not a substrate for this transferase?

It is considered of interest to entertain the possibility that a Le^a individual can possess the αLFuc transferase required to form the Le^d determinant but this enzyme is not expressed because the individual does not possess the $\beta\text{DGlcNAc}$ amidase required for the formation of the Type 1 disaccharide precursor in the amine form (Fig. 21). It would be expected that should, in fact, this amine be a precursor to the Le^d determinant, it would be formed either by N-deacetylation of the Type 1 disaccharide unit or by N-deacetylation of the precursor $\beta\text{DGlcNAc}$ unit for the formation of the Type 1 disaccharide unit as the amine. This latter postulation is considered of interest since it raises the possibility that the Type 1 and Type 2 structures are synthesized by way of the same βDGal transferase. As seen in Fig. 21, the steric environment about the 3-hydroxyl group of a $\beta\text{-D-glucosamine}$ unit bears a similarity to the steric environment about the 4-hydroxyl group of an N-acetyl- $\beta\text{-D-glucosamine}$ unit. The difference is the difference between a CH_2 group and a NH_2 group. This difference may prove to not be highly significant in terms of binding to the enzyme active site.

It could be anticipated on theoretical grounds that intramolecular hydrogen bonding in a carbohydrate antigenic determinant could play an important role in determining

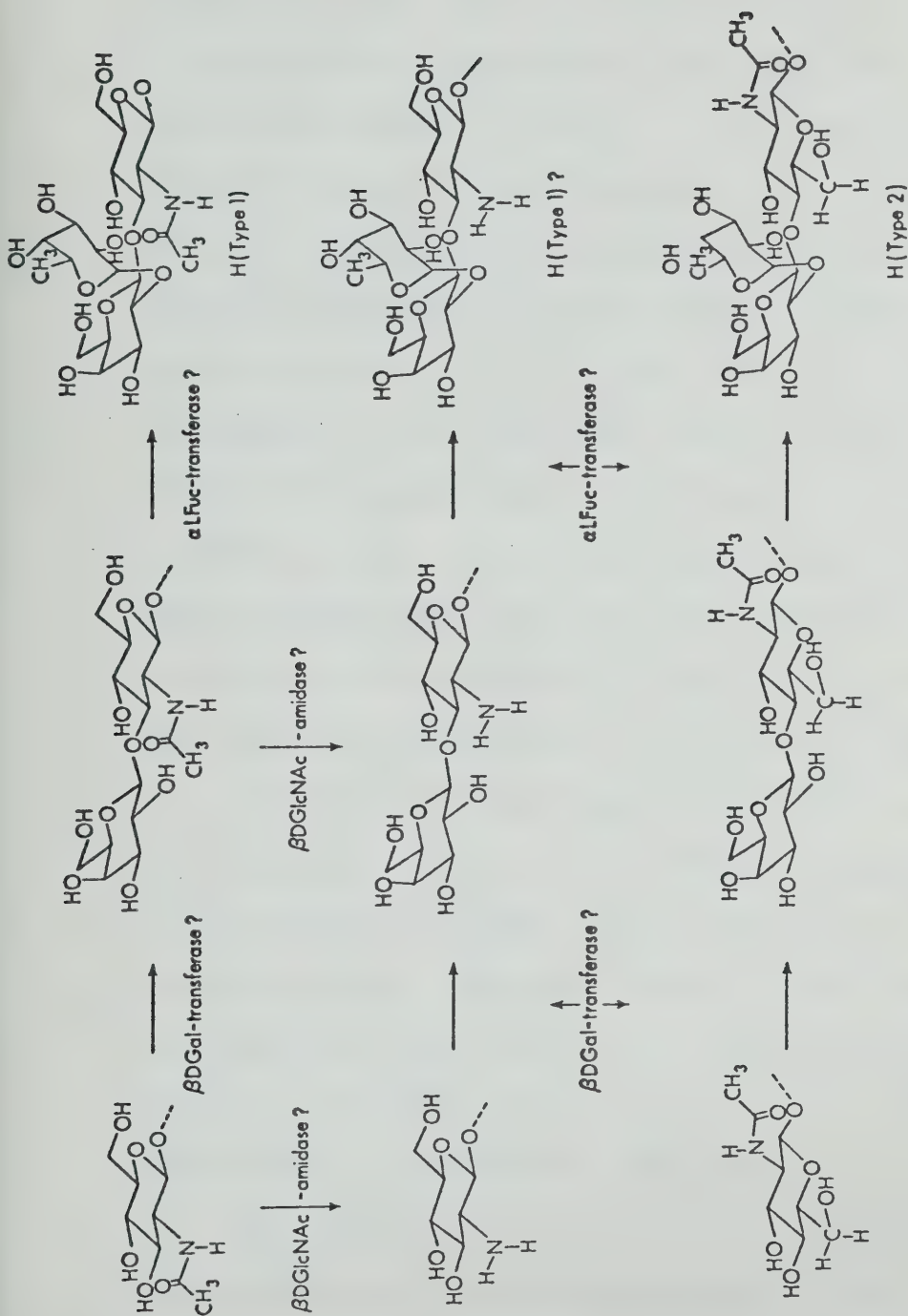


Fig. 21 Conformational formulas to illustrate the hypothesis, based in a suggested stereochemical equivalence for NH_2 and CH_2 groups, that the core disaccharides for the Type 1 and Type 2 human blood group determinants are synthesized by way of the same β DGal transferase and the Le^d and H determinants by the same α LFuc transferase. Should this be the case, as is illustrated, the Le^d determinant would be formed as the free amine rather than the N-acetylated form.

the energetics of the complexing of a carbohydrate with the combining site of an antibody, especially, should the driving force for binding be of an hydrophobic nature. In the course of recent studies,¹³ it was found that methyl 4-amino-4-deoxy- β -D-galactopyranoside was bound surprisingly well to anti- β -D-galactopyranosyl antibodies as compared to methyl 4-chloro-4-deoxy- β -D-galactopyranoside. Thus, it became apparent that the amino group, in a sense, can be isomorphous to an hydroxyl group. Meanwhile, Salmon and Gerbal¹¹⁴ showed that N-deacetylation of the β DGalNAc residue of the terminal trisaccharide of the A human blood group determinant rendered the structure B blood group active and thereby demonstrated that, in this interaction, the amine is in a sense isomorphous to the alcohol.

Evidence is thus accumulating that an amino group and an hydroxyl group can, in a sense, be isomorphous in terms of biological receptor sites. Especially if the binding is hydrophobic, an amine group can also be expected to be acceptable in the region of a receptor site which accommodates a methylene group. It was in the context of this idea that the situation presented in Fig. 22 was considered.

It was expected that, should the binding of the H determinant by the Ulex europaeus involve substantial binding of the β DGal unit, the e-H structure would inhibit the

agglutination of H red cells by this lectin much less effectively.

As seen in Fig. 22, J. LePendu found the H hapten to be a highly effective inhibitor of the Ulex agglutination of H human red cells. Furthermore, the e-H hapten in the same ester form was observed to be essentially as powerful an inhibitor. Subsequently, the N-deacetylated form of the H hapten was prepared, as the carboxylate salt, using the conditions prescribed by Lindberg and co-workers,¹¹⁵ and was found to be as effective an inhibitor as the electrically neutral structure. Therefore, it was apparent that the N-acetyl group is not involved in the binding of the H determinant with Ulex. As seen from Fig. 22, the Type 2 disaccharide was ineffective as an inhibitor both as the N-acetyl-methyl ester and as aminocarboxylate. Thus, it seemed clear that the binding site of the Ulex is directed largely to the α LFuc unit. Indeed, as seen in Fig. 22, the disaccharide α LFuc(1 \rightarrow 2) β DGal structure proved to be a quite good inhibitor. It was apparent therefore that, as previously appreciated,¹¹⁶ the Ulex combining site is directed toward the α LFuc group of the H determinant.

The observations presented in Fig. 22 require that the binding by Ulex is with that portion of the fucosyl group which faces the hydroxymethyl group of the β DGlcNAc

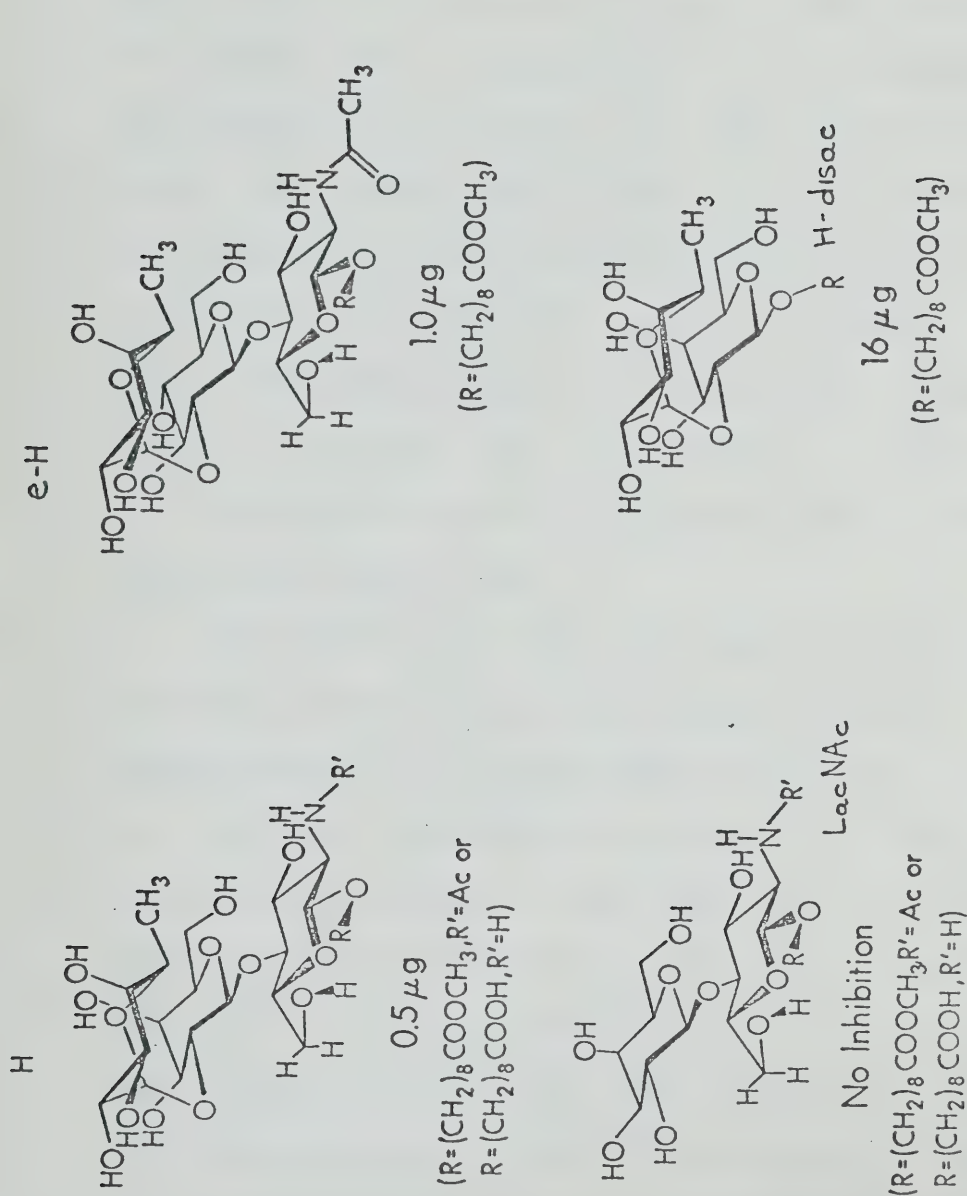


Fig. 22 The amounts of the structures shown required to inhibit the agglutination of HLe^a human red cells by the lectin Ulex europaeus in a total volume of 150 μL. It is concluded from these data that the Ulex combining site is directed about the C-3 - C-4 region of the αLFuc residue and may include binding with the CH₂OH region of the βDGlcnAc unit.

residue. On the basis of the conformational studies presented earlier, the indication is that the binding is in part with the region about the C-3 and C-4 positions of the α LFuc unit. A comparison of the conformational formulas presented in Fig. 23 for the H and Le^d structures shows that should this in fact be the case then, indeed, the acetamido group would interfere with the binding of Ulex with the α LFuc unit since it occupies a substantial space adjacent to the C-3 - C-4 region of the fucosyl group. Following this observation, it could be expected that the N-deacetylation could provide a structure with which Ulex would bind.

As seen in Fig. 23, the N-deacetylated Le^d trisaccharide proved to be an excellent inhibitor. The N-deacetylated Le^b tetrasaccharide structure was also a good but somewhat less effective inhibitor. Thus, the possibility exists that, in fact the Le^d and Le^b antigens are secreted in the amine form. If so, this would provide an explanation for the correlation between ABH secretor status and the distribution and expression of the gene responsible for the synthesis of the Le^d determinant. As was speculated with reference to Fig. 21, the genetic requirement may not be related to an α LFuc transferase but, instead to the availability of the Type 1 disaccharide unit in the amine form.

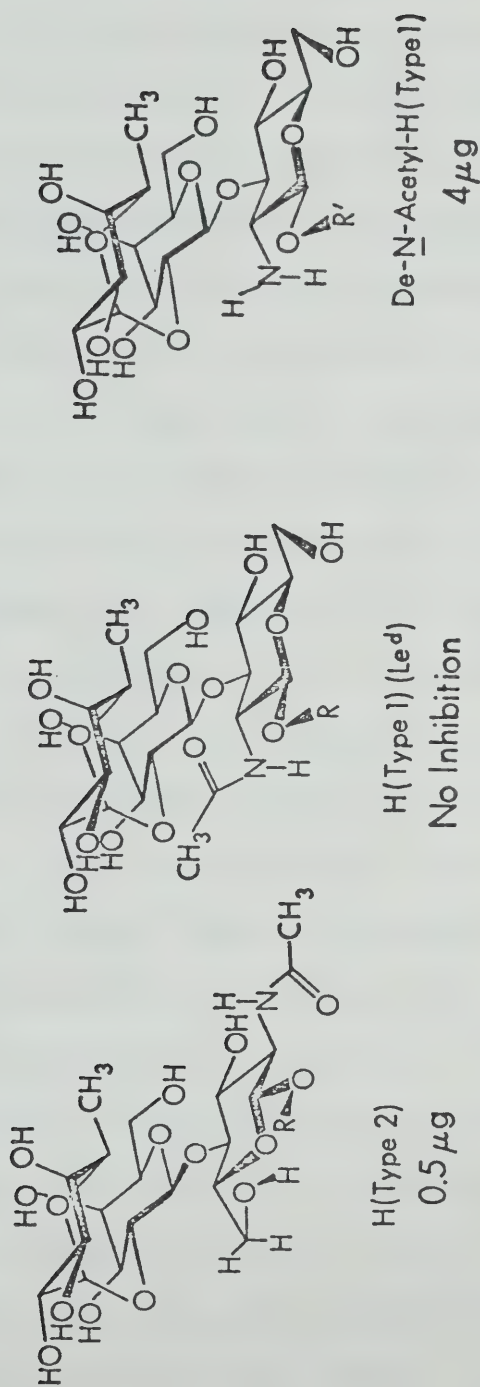


Fig. 23 Conformational formulas to indicate that, in accord with the data presented in Fig. 7, the Le^{d} determinant should not bind with Ulex since the acetamido group shields the C-3 - C-4 region of the αLFuc unit. Removal of the acetyl group results in a structure which has this region stereochemically similar to that in the H determinant which is well-bound by Ulex.

In order to gain further experimental justification for the hypothesis that the Ulex active substances in the secretions of H individuals have the Le^d and Le^b determinants in the amine form, the effect of N-acetylation of saliva on its ability to inhibit the agglutination of H red cells by Ulex was examined by Lemieux and LePendou. Conditions were found (acetic anhydride-methanol-water) which, when applied to the saliva of an A_1 secretor, caused no change in the inhibition of the agglutination of A human red cells by anti-A typing reagent by the saliva. However, the same acetylation of the saliva of an HLe^b person strongly reduced the inhibition of the agglutination of H human red cells by Ulex. The reduction was not quantitative (~75%), however, this may be due to an unusually high resistance of the amino groups of the Le^b determinant to acetylation. This is considered a possibility because the synthetic Le^d and Le^b haptens proved to be extremely resistant to N-deacetylation. Thus, using the conditions prescribed by Lindberg and co-workers,¹¹⁵ which employ 2.5 N sodium hydroxide, dimethylsulfoxide and thiophenol, the N-deacetylation of the simple $\beta DGlcNAC-O(CH_2)_8COO^-$ compound was complete within 5 hours at 100°C. In contrast, the quantitative N-deacetylation of the Le^d and Le^b haptens required nearly 65 hours at 120°C. No observable (pmr of the isolated product) deacetylation occurred after 5 hours

at 100°C. In control experiments, wherein acetic acid was substituted for the acetic anhydride, the treatment caused no reduction in the inhibition provided by the saliva.

Thus, the circumstantial evidence in support of the proposal that the Le^d and Le^b antigens in secretions have the determinant in the amine form appears to warrant serious attention.

CHAPTER V

EXPERIMENTAL

A. Synthetic Chemistry

All solvents and reagents were purified according to standard procedures.¹¹⁷ All solid reactants for glycosylation were dried overnight over phosphorous pentoxide in a high vacuum prior to use. The molecular sieve (BDH 4 \AA) was dried at 180°C for 24 h just prior to use. Solution transfers in these reactions were done under dry nitrogen using standard syringe technique.¹¹⁸ Removal of O-acetyl and O-p-nitrobenzoyl protecting groups was effected using a 0.02M solution of sodium methoxide in dry methanol.

The solutions obtained in the course of solvent extractions were filtered through paper pre-wetted with the solvent and further dried over sodium sulfate before solvent removal with a rotary evaporator under the vacuum of a water aspirator and, unless otherwise indicated, at a bath temperature of 35°C or less. Decolorization of residues were performed using a short column (2 cm in diameter) of neutral alumina (ca. 10 g) and using the solvent system described.

Thin layer chromatograms (TLC) were performed on pre-coated silica gel 60-F254 plates (E. Merck, Darmstadt) and visualized by quenching of fluorescence and/or by charring

after spraying with 5% sulphuric acid in ethanol. For column chromatography, silica gel H (type 60) (E. Merck, Darmstadt) and distilled solvents were used and the columns were loaded in the range 1:50 - 1:100.

Unless otherwise stated, proton magnetic resonance (^1H nmr) spectra were recorded on a Varian HA-100 or Brüker WH-200 at ambient temperature. Carbon-13 nuclear magnetic resonance (^{13}C nmr) spectra were recorded on a Brüker WP-60 (15.08 MHz) or HFX-90 (22.6 MHz) at ambient temperature. ^1H and ^{13}C nmr chemical shifts are in ppm relative to internal 1% tetramethylsilane (TMS) in organic solvents and either internal 1% acetone (δ 2.48) or internal 5% dioxane (δ 76.400) when the solvent was deuterium oxide. Optical rotations were measured on a Perkin-Elmer 241 Polarimeter at 589 nm in a 1 dm cell. The melting points are uncorrected.

8-Methoxycarbonyloctyl 2-Acetamido-3-O-(2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl)-4,6-O-benzylidene-2-deoxy- β -D-glucopyranoside (40)

To a stirred suspension of 8-methoxycarbonyloctyl 2-acetamido-4,6-O-benzylidene-2-deoxy- β -D-glucopyranoside¹⁰ (39) (7.25 g, 15.1 mmol), mercuric cyanide (7.84 g, 31.1 mmol) and powdered calcium sulfate (Drierite, 8.5 g) in a mixture of 1:1 benzene-nitromethane (180 mL) kept at 55°C, there was added 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide⁶⁷ (7) (7.44 g, 18.1 mmol) in 60 mL of the same solvent mixture. After 2.5 h, the reaction was cooled, filtered through a pad of Celite and then the filtrate made up to 1 L with dichloromethane. The solution was washed successively with saturated sodium bicarbonate (500 mL), saturated sodium chloride (500 mL) and water and then dried. Evaporation left a yellow syrup which was dissolved in ethylacetate-diethyl ether (3:1) and decolorized. Evaporation of the eluate left a white solid (11.39 g, 93%) which consisted of highly pure 40 which was crystallized in 77% yield by slow diffusion of pentane into a chloroform solution, mp 140-141, $[\alpha]_D^{25}$ -13.2 (C 1.1 CHCl₃); ¹Hnmr (CDCl₃) δ : 7.52-7.38 (m, 5H, aromatic), 5.94 (d, 1H, NH), 5.54 (s, 1H, benzylidene), 5.21 (d, $J_{1,2}$ = 8.5 Hz, 1H, H-1), 5.14-4.70 (m, 5H, H-1' (δ 4.78, $J_{1',2'}$ = 8.0 Hz), H-2', H-3', H-4', H-3), 4.33 (dd, $J_{5,6e}$ = 5.0 Hz, $J_{6a,6e}$ = 10.0 Hz, 1H, H-6_e), 4.12-3.31 (m, 11 H,

remaining sugar, OCH_3 (δ 3.65) and aglyconic), 3.02 (m, 1H, H-2), 2.31 (t, 2H, CH_2CO), 2.03, 2.00, 1.98, 1.92 (all s, 15H, COCH_3), 1.70-1.20 (m, 12H, aliphatic); $^{13}\text{Cnmr}$ (CDCl_3) δ : 174.22 (COOCH_3), 170.81, 170.52, 170.11, 169.52, 169.33 (C=O), 137.37 (quat. aromatic), 129.23, 128.26, 126.22 (remaining aromatic), 101.46, 99.85, 99.72 (C-1, C-1', benzylidene), 80.46 (C-4), 77.03, 73.07, 71.80, 71.61, 70.10, 68.77, 68.47, 66.00, 61.96 (remaining sugar and aglyconic: 9 lines expected, 9 lines found), 57.91 (C-2), 51.40 (OCH_3), 34.03 (CH_2CO), 29.47, 29.09, 28.99, 25.76, 24.88 (aliphatic), 23.51 (NHCOCH_3), 20.65, 20.52 (OCOCH_3). Anal. calcd. for $\text{C}_{39}\text{H}_{55}\text{O}_{17}\text{N}$: C 57.84, H 6.85, N 1.73; found: C 57.58, H 6.72, N 1.50.

8-Methoxycarbonyloctyl 2-Acetamido-3-O-(2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl)-2-deoxy- β -D-glucopyranoside (41)

A solution of compound 40 (5.37 g, 6.63 mmol) in 50% aqueous acetic acid (125 mL) was kept at 80°C for 30 min and the solvent was then evaporated (45°C bath). Traces of acetic acid were removed by the addition and subsequent evaporation of 4 portions of p-dioxane (100 mL). The residue was purified by column chromatography using dichloromethane-ethylacetate-n-hexane-ethanol (10:5:5:2) as eluent. The title compound was obtained as a white solid (4.11 g, 86%); $[\alpha]_D^{25}$ -3.9 (C 1.2 CHCl₃), ¹Hnmr (CDCl₃/CD₃OD, 1:1, 55°C) δ : 5.31-4.86 (m, 3H, H-2', H-3', H-4'), 4.70 (d, $J_{1',2'} = 8.5$ Hz, 1H, H-1'), 4.54 (d, $J_{1,2} = 8.0$ Hz, 1H, H-1), 4.32-3.66 (m, remaining sugar, OCH₃ (δ 3.66) and aglyconic), 2.30 (t, 2H, CH₂CO), 2.08, 2.06, 2.04, 2.00 (all s, 15H, COCH₃), 1.80-1.20 (m, 12H, aliphatic): ¹³Cnmr (CDCl₃) δ : 174.36 (COOCH₃), 170.90, 170.60, 170.09, 169.44, 169.31 (C=O), 101.01, 99.83 (C-1, C-1'), 84.18 (C-4), 75.28, 72.80, 71.72, 71.40, 70.06, 69.91, 68.51 (ring and aglyconic: 7 lines expected, 7 lines found), 62.52, 62.06 (C-6, C-6'), 56.59 (C-2), 51.46 (OCH₃), 34.04 (CH₂CO), 29.48, 29.33, 29.13, 29.00, 25.81, 24.87 (aliphatic), 23.57 (NHCOCH₃), 20.72, 20.52 (OCOCH₃). Anal. calcd. for C₃₂H₅₁O₁₇N: C 53.25, H 7.12, N 1.94; found: C 53.15, H 7.02, N 1.84.

8-Methoxycarbonyloctyl 2-Acetamido-6-O-acetyl-3-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (42)

A solution of acetyl chloride (0.45 mL, 6.29 mmol) in dichloromethane (7 mL) was added dropwise, over a period of 10 min, to a stirred solution of 41 (3.78 g, 5.24 mmol) and pyridine (6.3 mmol) in dichloromethane (60 mL) kept at -78°C. After 10 min, the reaction was poured into water (100 mL) and extracted with dichloromethane (100 mL). The organic phase was washed twice with water, dried and evaporated to provide 42 as a chromatographically homogeneous syrup (3.87 g, 97%) which crystallized from acetone-n-hexane (71%); mp 155-156, $[\alpha]_D^{25} -5.3$ (C 1.0 CHCl₃); ¹Hnmr (CDCl₃/CD₃OD, 1:1, 32°C) δ: 5.34-4.89 (m, 3H, H-2', H-3', H-4'), 4.89 (d, J_{1',2'} = 8.0 Hz, 1H, H-1'), 4.69 (d, J_{1,2} = 8.5 Hz, 1H, H-1), 4.40-3.30 (m, remaining sugar, OCH₃ (δ3.66) and aglyconic), 2.33 (t, 2H, CH₂CO), 2.11, 2.08, 2.06, 2.02 (all s, 18H, COCH₃), 1.80-1.20 (m, 12H, aliphatic); ¹³Cnmr (CDCl₃) : 174.38 (COOCH₃), 170.89, 170.60, 170.11, 169.44, 169.21 (C=O), 101.03, 99.27 (C-1, C-1'), 83.50 (C-4), 73.48, 72.80, 71.93, 71.46, 69.81, 69.70, 68.59 (ring and aglyconic: 7 lines expected, 7 lines found), 63.67, 62.07 (C-6, C-6'), 57.39 (C-2), 51.43 (OCH₃), 34.08 (CH₂CO), 29.51, 29.08, 25.80, 24.89 (aliphatic), 23.56 (NHCOCH₃), 20.81, 20.51 (OCOCH₃). When the ¹³Cnmr spectrum

was recorded using $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1 as solvent, all 7 carbonyls were observed: δ : 175.29, 172.12, 171.88, 171.51, 170.90, 170.41, 170.30. Anal. calcd. for $\text{C}_{34}\text{H}_{53}\text{O}_{18}\text{N}$: C 53.47, H 6.99, N 1.83; found: C 53.21, H 6.92, N 2.02.

8-Methoxycarbonyloctyl 2-Acetamido-6-O-acetyl-3-O-(2,3, 4, 6-tetra-O-acetyl- β -D-glucopyranosyl)-4-O-(2,3,4-tri-O-benzyl- α -L-fucopyranosyl)-2-deoxy- β -D-glucopyranoside (44)

To a mixture of 42 (2.45 g, 3.21 mmol), tetraethylammonium bromide (0.73 g, 3.48 mmol), diisopropylethylamine (0.91 mL, 5.22 mmol), molecular sieve (3.8 g), dimethylformamide (1.4 mL) and dichloromethane (7 mL), there was added 2,3,4-tri-O-benzyl- α -L-fucopyranosyl bromide (43) [freshly prepared⁷¹ from 2,3,4-tri-O-benzyl-1-O-p-nitrobenzoyl- α,β -L-fucopyranose (2.50 g, 4.29 mmol)] in dichloromethane (2 mL). After 4 and 6 days, further amounts of 43 (2.50 g) were added. After a total of 7 days, TLC examination showed that no more starting material remained. The reaction was diluted with dichloromethane, filtered through a pad of Celite and the pad washed thoroughly with dichloromethane to a filtrate volume of 130 mL which was twice washed with water. Drying and evaporation left a brown residue which was dissolved in ethylacetate-diethyl ether (3:1) for decolorization. The pale yellow syrup obtained after evaporation of the eluent was purified by chromatography using dichloromethane-ethylacetate-n-hexane-ethanol (10:5:5:2) as eluent. Evaporation of the appropriate fractions (R_f 0.58) provided the pure trisaccharide derivative as a white foam (3.64 g, 96%); $[\alpha]_D^{25}$ -65.2 (C 1.1 CHCl_3)¹Hnmr (CDCl_3) δ : 7.48-7.04 (m, 15H, aromatic), 6.42 (d, 1H, NH), 5.26-3.22 (m, 30H, remaining sugar, OCH_3 (δ 3.64),

benzyl and aglyconic), 2.29 (t, 2H, CH_2CO), 2.04, 2.00, 1.98 (all s, 15H, OCOCH_3), 1.68-1.16 (m, 18H, including NHCOCH_3 (s, δ 1.68 H-6" (d, $J_{5'',6''} = 6.5$ Hz) and aliphatic); $^{13}\text{Cnmr}$ (CDCl_3) : 174.19 (COOCH_3), 170.36, 170.00, 169.79, 169.41, 169.12 (C=O), 138.61, 138.53, 137.88 (quat. aromatic), 128.63, 128.44, 128.25, 128.00, 127.64, 127.15 (remaining aromatic), 99.29, 98.47 (C-1, C-1'), 94.58 (C-1"), 79.87, 77.51, 74.87, 74.12, 73.04, 72.77, 72.04, 70.99, 70.26, 68.83, 68.29, 67.03 (ring, aglyconic and benzylic: 15 lines expected, 12 lines found), 63.43, 61.77 (C-6, C-6'), 51.35 (C-2 and OCH_3), 34.04 (CH_2CO), 29.39, 29.23, 29.13, 29.07, 26.02, 24.91 (aliphatic), 22.89 (NHCOCH_3), 20.87, 20.63, 20.52 (OCOCH_3), 16.74 (C-6"). Anal. calcd. for $\text{C}_{61}\text{H}_{81}\text{O}_{22}\text{N}$: C 62.07, H 6.92, N 1.19; found C 61.91, H 6.98, N 1.06.

8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-4-O-(α -L-fucopyranosyl)-3-O-(β -D-glucopyranosyl)- β -D-glucopyranoside (2)

A solution of 44 (3.10 g, 2.62 mmol) in dry methanol (45 mL) containing a catalytic amount of sodium methoxide was kept at room temperature for 12 h prior to neutralization with Amberlite 1R-120(H⁺). Removal of the resin and evaporation provided the de-O-acetylated tri-saccharide derivative (quantitative yield). This product was then dissolved in 96% ethanol (55 mL) containing 5% palladium on charcoal (2.50 g) and the mixture was kept stirring under a 200 psi atmosphere of hydrogen for 48 h at room temperature. The catalyst was removed by filtration through Celite and washed with several portions of hot ethanol. Evaporation of the filtrate gave the title compound as a white glass (1.58 g, 86%); $[\alpha]_D^{25}$ -79.1 (C 1.1 H₂O). The ¹H and ¹³Cnmr parameters are reported in Tables 1 and 2, respectively.

Treatment of 2 (360 mg) with 85% hydrazine hydrate-ethanol 4:1 (10 mL) at room temperature for 6 h, followed by evaporation to dryness and the addition and evaporation of 3 portions of n-butanol (10 mL) provided the hydrazide in quantitative yield. The ¹Hnmr of this hydrazide was identical to that of 2 except for a 0.17 ppm upfield shift for the CH₂CO protons and the disappearance of signal for the methoxy protons.

3,4,6-Tri-O-benzyl-1,2-O-(1-ethoxyethylidene)

- α -D-glucopyranose (50)

A solution of 3,4,6-tri-O-acetyl-1,2-O-(1-ethoxyethylidene)- α -D-glucopyranose (74) (20.68 g, 54.9 mmol) in dry methanol (150 mL) containing a catalytic amount of sodium methoxide was kept at room temperature for 12 h and evaporated. The residual dry syrup was dissolved in dimethylformamide (100 mL) and sodium hydride (7.63 g of a 56-58% dispersion in oil) was added in portions and, after evolution of hydrogen had ceased, benzyl bromide (21.5 mL, 180 mmol) was added dropwise, with cooling, to the resulting slurry. After 15 h, when TLC examination showed benzylation to be complete, methanol (10 mL) was added to destroy excess reagent. After 2 h, the mixture was diluted with diethyl ether (500 mL), filtered through a Celite pad and washed 4 times with water (500 mL) before drying and evaporation. Chromatography of the residue, using ethylacetate-n-hexane 2:1, containing 0.1% triethylamine, as eluent provided 50 as a clear syrup (24.0 g, 84%) which resisted crystallization; $[\alpha]_D^{22}$ 34.4 (C 1.1, CHCl_3); $^1\text{Hnmr}$ (CDCl_3) δ : 7.65-7.00 (m, 15H, aromatic), 5.73 (d, $J_{1,2}$ = 5.5 Hz, 1H, H-1), 4.76-4.28 (m, 7H, H-2 and benzyl), 3.96-3.34 (m, 7H, remaining sugar and methylene), 1.62 (s, 3H, CH_3), 1.16 (t, 3H, OCH_2CH_3); $^{13}\text{Cnmr}$ (CDCl_3) δ : 138.28, 138.16, 137.92 (quat.

aromatic), 128.97, 128.48, 128.37, 128.05, 127.88, 127.34
(remaining aromatic), 121.12 (quat. orthoester), 97.87
(C-1), 79.07, 76.02, 75.11, 73.46, 72.98, 71.95, 70.67,
69.33 (remaining sugar and benzylic: 8 lines expected,
8 lines found), 58.61 (OCH_2CH_3), 21.97 (CH_3), 15.34
(OCH_2CH_3).

2-O-Acetyl-3,4,6-tri-O-benzyl- α -D-glucopyranosyl bromide (53)

A mixture of 50 (2.21 g, 4.25 mmol), tetramethylammonium bromide (0.42 g, 2.01 mmol), molecular sieve (3.3 g) and dichloromethane (16 mL) was left stirring at room temperature for 2 h before acetyl bromide (0.60 mL, 8.2 mmol) was added. After 1 h TLC examination, using benzene-ethylacetate 4:1 as irrigant, showed almost exclusive conversion of 13 (Rf 0.50) to a more mobile product (Rf 0.55). The reaction was then poured into a vigorously stirring mixture of saturated sodium bicarbonate, ice and dichloromethane and filtered to remove the sieves. The organic phase was washed with cold bicarbonate, water and dried before evaporation to a clear syrup. The nmr spectra of this product showed it to consist of over 90% of 14: $^1\text{Hnmr}$ (CDCl_3) δ : 7.40-7.10 (m, 15H, aromatic), 6.63 (d, $J_{1,2} = 4.0$ Hz, $\geq 0.9\text{H}$, H-1), 4.90-4.37 (m, 7H, H-2 and benzylic), 4.16-3.55 (m, 5H, remaining sugar), 2.00 (s, $\geq 2.8\text{H}$, COCH_3); $^{13}\text{Cnmr}$ (CDCl_3) : 169.87 (C=O), 138.34, 137.97, 137.90 (quat. aromatic), 128.48, 127.89, 127.64 (remaining aromatic), 89.53 (C-1), 80.40, 76.37, 75.53 (2C), 75.29, 73.52, 73.40, 67.61 (remaining sugar and benzylic: 8 lines expected, 7 lines found), 20.69 (COCH_3).

8-Methoxycarbonyloctyl 2-Acetamido-3-O-(2-O-acetyl-3,4,6-tri-O-benzyl- β -D-glucopyranosyl)-4,6-O-benzylidene-2-deoxy- β -D-glucopyranoside (54)

Compound 39 (1.02 g, 2.12 mmol) was engaged for 2 h in the Helferich reaction with 53 (1.25 g, 4.9 mmol) under the conditions described for the preparation of 40. Processing, as before, followed by chromatography, using ethylacetate-n-hexane (3:2) as eluent, provided the pure title disaccharide derivative (R_f 0.41) as a clear syrup (1.16 g, 57%) which crystallized from ethylacetate-n-hexane; mp 150-151, $[\alpha]_D^{25}$ -1.3 (C 1.0 CHCl_3); $^1\text{Hnmr}$ (CDCl_3) δ : 7.41-7.12 (m, 20H, aromatic), 5.92 (d, 1H, NH), 5.36 (s, 1H, benzylidene), 5.20 (d, $J_{1,2} = 8.0$ Hz, 1H, h-1), 4.93 (t, $J_{1',2'} = J_{2',3'} = 8.5$ Hz, 1H, H-1'), 4.79-4.46 (m, 8H, H-1', h-3 and benzylic), 4.28 (q, $J_{5,6e} = 4.5$ Hz, $J_{6a,6e} = 10.0$ Hz, 1H, H-6_e), 3.90-2.98 (m, 14H, remaining sugar, OCH_3 (δ 3.69) and aglyconic), 2.31 (t, 2H, CH_2CO), 1.95, 1.86 (both s, 6H, COCH_3), 1.70-1.20 (m, 12H, aliphatic); $^{13}\text{Cnmr}$ (CDCl_3) : 174.31 (COOCH_3), 170.59, 169.62 (C=O), 138.25, 138.03, 137.49 (quat. aromatic), 129.51, 128.90, 128.47, 128.29, 128.04, 127.80, 126.34 (remaining aromatic), 101.58, 99.57 (2C) (C-1, C-1', benzylidene), 83.14 (C-4), 80.89, 78.02, 76.13, 74.98, 74.56, 73.92, 73.39, 70.22, 68.89, 68.70,

66.08 (remaining sugar, benzyl and aglyconic: 12 lines expected, 11 lines found), 58.66 (C-2), 51.42 (OCH₃), 34.07 (CH₂CO), 29.51, 29.08, 25.79, 24.89 (aliphatic), 23.61 (NHCOCH₃), 20.92 (OCOCH₃). Anal. calcd. for C₅₄H₆₇O₁₄N: C 67.98, H 7.08, N 1.47; found: C 67.84, H 7.11, N 1.33.

8-Methoxycarbonyloctyl 2-Acetamido-3-O-(3,4,6-tri-O-benzyl-
 β -D-glucopyranosyl)-4,6-O-benzylidene-2-deoxy- β -D-
glucopyranoside (55)

A solution of 54 (600 mg, 0.63 mmol) in warm methanol (20 mL) containing a catalytic amount of sodium methoxide was kept at room temperature overnight. Neutralization and evaporation provided 55 in quantitative yield; $[\alpha]_D^{25} -18.5$ (C 1.4 CHCl_3); $^1\text{Hnmr}$ (CDCl_3) δ : 7.52-7.01 (m, 20 H, aromatic), 6.07 (d, 1H, NH), 5.54 (s, 1 H, benzylidene), 4.83-4.30 (m, 9 H, benzyl, H-1 (δ 4.72, $J_{1,2} = 7.5$ Hz), H-1' and H-6_e), 4.22 (t, 1H, H-3), 3.90-3.36 (m, 15 H, sugar, OCH_3 (δ 3.66) and aglyconic), 3.12 (br, 1 H, OH), 2.29 (t, 2 H, CH_2CO), 1.88 (s, 3 H, NHCOCH_3), 1.70-1.20 (m, 12 H, aliphatic).

8-Methoxycarbonyloctyl 2-Acetamido-3-O-[3,4,6-tri-O-benzyl-
2-O-(2,3,4-tri-O-benzyl- α -L-fucopyranosyl)-4,6-O-benzylidene-
2-deoxy- β -D-glucopyranoside (56)

Compound 55 (600 mg, 0.63 mmol) and the fucosyl bromide 43 (2.4 eq.) were engaged in the halide-ion catalyzed reaction under the conditions described for the preparation of 44, except that no further additions of 43 were made. After 41 h, the reaction mixture was processed as described for 44 and, after the decolorization, the residue was chromatographed using n-hexane-ethylacetate (3:2) as eluent. Evaporation of the trailing fractions gave back unreacted 55 (222 mg, 38%). Rechromatography of the intermediate fractions using ethylacetate-n-hexane-dichloromethane (2:2:1) provided the title compound (R_f 0.50) as a white foam (297 mg, 37%); $[\alpha]_D^{22}$ -40.8 (C 1.0, CHCl₃); $^1\text{Hnmr}$ (CDCl₃) δ : 7.44-7.04 (m, 35H, aromatic), 6.48 (d, 1H, NH), 5.39 (2H, benzylidene and H-1'', $J_{1'',2''} \leq 4.0$ Hz), 5.08-4.41 (m, 14H, H-1, H-1' and benzylidene), 4.30-4.14 (m, 3H, H-3, H-5'' and H-4?), 4.01 (q, $J_{5,6e} = 4.0$ Hz, $J_{6a,6e} = 10.5$ Hz, 1H, H-6e), 3.89-3.12 (m, 17H, remaining sugar, OCH₃ (δ 3.68) and aglyconic), 2.30 (t, 2H, CH₂CO), 1.74 (s, 3H, NHCOCH₃), 1.70-1.10 (m, 15H, aliphatic and H_{6''} (δ 1.20), $J_{5'',6''} = \text{ca. } 7.5$ Hz); $^{13}\text{Cnmr}$ (CDCl₃) δ : 174.31 (COOCH₃), 170.48 (NHCOCH₃), 138.89, 138.71, 138.47, 138.34, 137.56 (quat. aromatic), 130.19,

129.82, 129.64, 129.08, 128.35, 128.06, 127.64, 127.44, 127.10, 126.84, 126.65, 126.36 (remaining aromatic), 101.78 (2C), 101.56 (C-1, C-1', benzylidene), 97.75 (C-1"), 85.14 (C-4), 80.04, 79.54, 77.85, 76.89, 76.21, 74.93, 74.56, 73.54, 72.98, 70.00, 68.90, 67.43, 66.57 (remaining sugar, benzyl and aglyconic: 19 lines expected, 13 lines found), 57.04 (C-2), 51.37 (OCH₃), 34.09 (CH₂CO), 29.58, 29.15 25.80, 24.95 (aliphatic), 23.13 (NHCOCH₃), 16.97 (C-6"). Anal. calcd. for C₇₈H₉₃O₁₇N: C 71.16, H 7.11, N 1.06; found: C 71.21, H 7.01, N 0.97.

8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-3-O-[2-O-(α -L-fucopyranosyl)- β -D-glucopyranosyl]- β -D-glucopyranoside (4)

Compound 56 (119 mg, 0.090 mmol) was dissolved in 95% ethanol (10 mL) containing 5% palladium on charcoal (120 mg) and was hydrogenated at 140 psi for 40 h. Examination by TLC at this point showed only a single spot (R_f 0.57 in 2-propanol-water 8:2). Processing, as described for 2, gave the title compound (60 mg, 95%): $[\alpha]_D^{25} -76.2$ (C 1.0 H₂O). The ^1H and ^{13}C nmr parameters are reported in Tables 1 and 2, respectively.

Treatment of 4 with 85% hydrazine-hydrate-ethanol 3:2 (5 mL) at room temperature for 2 h resulted in its complete conversion to the hydrazide. This compound was isolated as described for the hydrazide of 2 and showed the equivalent changes in its ^1H nmr spectrum.

8-Methoxycarbonyloctyl 2-Acetamido-4,6-O-benzylidene-3-O-benzylloxymethyl-2-deoxy- β -D-glucopyranoside (57)

A solution of 8-methoxycarbonyloctyl 2-acetamido-4,6-O-benzylidene-2-deoxy- β -D-glucopyranoside (39) (18.4 g, 38.4 mmol), 2,6-lutidine (8.9 mL, 77 mmol) and benzyl chloromethyl ether (8.0 mL, 58 mmol) in dry acetonitrile (140 mL) was kept at 80°C for 12 h. Methanol (10 mL) was then added and, after 1 h, the solution was taken to dryness. The residue was dissolved in dichloromethane (400 mL) and washed with cold 2% HCl, saturated sodium hydrogen carbonate and water before drying and evaporation. The residual dark syrup was then dissolved in dichloromethane-ethylacetate (2:1) and decolorized.

Evaporation of the eluate and crystallization from methanol provided the title compound (18.2 g, 79%); m.p. 159 - 160 °C, $[\alpha]_D^{25}$ 8.4 (C 1.0 CHCl₃) ; ¹Hnmr (CDCl₃) δ : 7.49-7.22 (m, 10H, aromatic), 5.79 (d, 1H, NH), 5.50 (s, 1H, benzylidene), 5.00-4.77 (m, 3H, H-1(δ 4.84, $J_{1,2}$ = 8.0 Hz) and OCH₂O: AB, δ A = 4.93, δ B = 4.82, J_{AB} = 7.0 Hz), 4.72-4.44 (2H, benzyl: AB, δ A = 4.61, δ B = 4.56, J_{AB} = 12.0 Hz), 4.41-4.22 (m, 2H, H-3 and H-6_e), 3.99-3.44 (m, 9H, remaining sugar, OCH₃ (δ 3.64) and aglyconic), 2.29 (t, 2H, CH₂CO), 1.84 (s, 3H, NHCOCH₃), 1.70-1.20 (m, 12H, aliphatic); ¹³Cnmr (CDCl₃) δ : 174.23 (COOCH₃), 170.41 (NHCOCH₃), 137.89, 137.31 (quat.

aromatic), 128.88, 128.30, 128.14, 127.72, 127.48, 126.09
(remaining aromatic), 101.30 (C-1 and benzylidene), 81.79
(C-4), 75.75 (C-3), 69.93, 69.45, 68.73, 65.79 (C-5, C-6,
benzyl and aglyconic), 56.48 (C-2), 34.02 ($\underline{\text{CH}}_2\text{CO}$), 29.50,
29.09, 28.99, 25.75, 24.86 (aliphatic), 23.33 ($\text{NHCO}\underline{\text{CH}}_3$).

Anal. calcd. for $\text{C}_{33}\text{H}_{45}\text{O}_9\text{N}$: C 66.09, H 7.56, N 2.34;

found: C 66.22, H 7.60, N 2.14.

8-Methoxycarbonyloctyl 2-Acetamido-3-O-(benzyl-
oxymethyl)-2-deoxy- β -D-glucopyranoside (59)

Warm 50% aqueous acetic acid (80°C, 300 mL) was added to a solution of compound 57 (16.2 g, 27.0 mmol) in p-dioxane (50 mL) and the resulting solution was kept at 80°C for 75 min. The solvent was then evaporated and traces of acetic acid were removed by the addition and evaporation of 3 portions of p-dioxane (150 mL). The residue was purified by column chromatography using dichloromethane-n-hexane-ethylacetate-ethanol (10:5:5:2) as eluent. The title compound was obtained as an amorphous white powder from acetone-n-hexane (11.1 g, 80%), $[\alpha]_D^{25}$ 4.2 (C 1.2 CHCl₃); ¹Hnmr (acetone-d₆, D₂O exchanged sample) δ : 7.40-7.10 (m, 5H, aromatic), 5.01-4.74 (AB, δ A = 4.96, δ B = 4.79, J_{AB} = 6.7 Hz, 2H, OCH₂O), 4.63-4.51 (3H, H-1 and benzyl), 3.89-3.16 (m, 11H, sugar, OCH₃ (3.56) and aglyconic), 2.24 (t, 2H, CH₂CO), 1.78 (s, 3H, NHCOCH₃), 1.70-1.20 (m, 12H, aliphatic): ¹³Cnmr (CD₃OD) δ : 175.82 (COOCH₃), 173.17 (NHCOCH₃), 139.21 (quat. aromatic), 129.31, 128.70, 128.58 (remaining aromatic), 102.52 (C-1), 96.91 (OCH₂O), 83.07 (C-4), 77.65 (C-3), 71.97, 70.58, 70.51 (C-5, benzyl and aglyconic), 62.69 (C-6), 56.32 (C-2), 51.90 (OCH₃), 34.72 (CH₂CO), 30.56, 30.29, 30.22, 30.05, 26.97, 25.94 (aliphatic), 23.12 (NHCOCH₃).

Evaporation of the trailing fractions from the column provided 8-methoxycarbonyloctyl 2-acetamido-2-deoxy- β -D-glucopyranoside (58) (1.28g, 12%), identified by comparison with an authentic sample.¹⁰

8-Methoxycarbonyloctyl 2-Acetamido-3,6-di-O-benzyloxymethyl-
2-deoxy- β -D-glucopyranoside (60)

A solution of compound 59 (10.5 g, 20.5 mmol), benzyl chloromethyl ether (3.11 mL, 22.5 mmol) and 2,6-lutidine (3.6 mL, 31.0 mmol) in dimethylformamide (35 mL) was kept at room temperature for 16 h. Methanol (2 mL) was then added and, after 1 h, the solution was diluted with dichloromethane (300 mL) and washed with cold 2% HCl (300 mL), saturated sodium hydrogen carbonate (300 mL) and water, each time back extracting with dichloromethane (100 mL). Drying and evaporation left a white gum (11.6 g) which crystallized from ethanol-water (7.86 g, 61%). Chromatography of the mother liquor provided an additional 1.2 g (9%): m.p. 112 - 113, $[\alpha]_D^{25}$ -24.4 (C 1.4 CHCl₃); ¹Hnmr (CDCl₃) δ : 7.43-7.14 (m, 10H, aromatic), 5.82 (d, 1H, NH), 4.94-4.48 (m, 9H, benzyl, OCH₂O and H-1), 4.12-3.06 (m, 12H, remaining sugar, OCH₃ (δ 3.62), OH (br, δ 4.12), and aglyconic, 2.26 (t, 2H, CH₂CO), 1.89 (s, 3H, NHCOCH₃), 1.70-1.20 (m, 12H, aliphatic); ¹³Cnmr (CDCl₃) δ : 174.30 (COOCH₃), 170.57 (NHCOCH₃), 137.83, 136.81 (quat. aromatic), 128.55, 128.39, 127.85, 127.64 (remaining aromatic), 99.95 (C-1), 96.19, 94.78 (OCH₂O), 83.55 (C-4), 74.82 (C-3), 70.56, 70.24, 69.54, 69.21 (C-5, benzyl and aglyconic), 67.04 (C-6), 56.76 (C-2), 51.41 (OCH₃), 34.04 (CH₂CO),

29.50, 29.09, 28.98, 25.79, 24.87 (aliphatic), 23.47

(NHCOCH₃). Anal. calcd. for C₃₄H₄₉O₁₀N: C 64.64, H 7.82,
N 2.22; found: C 64.34, H 7.80, N 2.16.

8-Methoxycarbonyloctyl 2-Acetamido-4-O-(3,4,6-tri-O-benzyl- β -D-galactopyranosyl)-3,6-di-O-benzylloxymethyl-2-deoxy- β -D-glucopyranoside (62)

A solution of 3,4,6-tri-O-benzyl-2-O-p-nitrobenzoyl- α -D-galactopyranosyl bromide (47) [freshly prepared¹⁴ from 3,4,6-tri-O-benzyl-1,2-di-O-p-nitrobenzoyl- α,β -D-galactopyranose (46) (4.08 g, 5.45 mmol)] in dichloromethane (8 mL) was added to a solution of compound 60 (1.90 g, 3.01 mmol), silver trifluoromethanesulfonate (1.36 g, 5.29 mmol) and tetramethylurea (1.4 mL, 11.7 mmol) in dry dichloromethane (13 mL) and the mixture was stirred at room temperature, protected from light and moisture, for 42 h. The reaction mixture was then filtered through a pad of celite and the filtrate was made up to 125 mL with dichloromethane and washed once with saturated sodium hydrogen carbonate and twice with water prior to drying and evaporation. The residual brown syrup was chromatographed using ethylacetate-n-hexane (1:1) as eluent, providing two crude disaccharide containing fractions. The first fraction (Rf 0.55) proved to be a mixture of compounds and was de-O-p-nitrobenzoylated using sodium methoxide in methanol. The main component was isolated by chromatography, eluting first with dichloromethane, then dichloromethane-ethylacetate (1:1), providing 8-methoxycarbonyloctyl 2-acetamido-4-O-

(3,4,6-tri-O-benzyl- α -D-galactopyranosyl)-3,6-di-O-benzyl-oxymethyl-2-deoxy- β -D-glucopyranoside as a clear syrup (661 mg, 21% from 60).

$^1\text{Hnmr}$ (CDCl_3) δ : 5.17 (d, $J_{1',2'} = 3.5$ Hz, 1H, H-1); $^{13}\text{Cnmr}$ (CDCl_3) δ : 99.96, 99.15 (C-1, C-1'), 95.16, 94.69 (OCH_2O).

Evaporation of the second crude fraction (Rf 0.30) provided slightly impure 8-methoxycarbonyloctyl 2-acetamido-4-O-(3,4,6-tri-O-benzyl-2-O-p-nitrobenzoyl- β -D-galactopyranosyl)-3,6-di-O-benzylloxymethyl-2-deoxy- β -D-glucopyranoside (61) (1.31 g, 36%): $^1\text{Hnmr}$ (CDCl_3) δ : 5.54 (dd, $J_{1',2'} = 8.0$ Hz, $J_{2',3'} = 9.5$ Hz, 1H, H-2'); $^{13}\text{Cnmr}$ (CDCl_3) δ : 100.55, 100.40 (C-1, C-1'), 95.65, 94.94 (OCH_2O). De-O-p-nitrobenzoylation of 61, followed by chromatography as described for the α -anomer, provided the title compound (62) which crystallized from ethanol (787 mg, 25%): m.p. 108-109, $[\alpha]_D^{25} +2.90$ (C 0.7 CHCl_3); $^1\text{Hnmr}$ (CDCl_3) δ : 7.36, 7.22 (m, 25H, aromatic), 5.66 (d, 1H, NH), 4.95-4.29 (m, 16H, benzyl, H-1 and H-1'), 4.01-3.32 (m, 17H, sugar, OCH_3 (δ 3.64) and aglyconic), 2.26 (t, 2H, CH_2CO), 1.60-1.20 (m, 16H, including NHCOCH_3 (δ 1.70), OH and aliphatic). Anal. calcd. for $\text{C}_{61}\text{H}_{77}\text{O}_{15}\text{N}$: C 68.84, H 7.29, N 1.32; found: C 68.65, H 7.10, N 1.36.

8-Methoxycarbonyloctyl 2-Acetamido-4-O-[3,4,6-tri-O-benzyl-2-O-(2,3,4-tri-O-benzyl- α -L-fucopyranosyl)- β -D-galactopyranosyl]-3,6-di-O-benzyloxymethyl-2-deoxy- β -D-glucopyranoside (64)

To a mixture of compound 62 (633 mg, 0.59 mmol), tetraethylammonium bromide (185 mg, 0.88 mmol), diisopropylethylamine (0.28 mL, 1.60 mmol), molecular sieve (830 mg), dimethylformamide (0.50 mL) and dichloromethane (2.5 mL), there was added 2,3,4-tri-O-benzyl- α -L-fucopyranosyl bromide (43) [freshly prepared from 2,3,4-tri-O-benzyl-1-O-p-nitrobenzoyl- α , β -L-fucopyranose¹⁴ (905 mg, 1.55 mmol)] in dichloromethane (1.0 mL). After stirring at room temperature for 26 h, the reaction mixture was diluted with dichloromethane, filtered through Celite and the filtrate was made up to 60 mL with dichloromethane. Washing twice with water, followed by drying and evaporation, left a brown syrup which was purified with chromatography, eluting first with *n*-hexane-ethylacetate (2:1), then dichloromethane-ethylacetate (1:1), and provided a trisaccharide containing fraction (765 mg). The nmr spectra of this material showed it to be a mixture of two compounds and chromatography using dichloromethane-ethylacetate (4:1) separated the components. Evaporation of the latter fraction (R_f 0.49) provided the title compound (385 mg, 44%) as a clear syrup: $[\alpha]_D^{25}$ -28.2 (C 0.6 CHCl₃); ¹Hnmr (CDCl₃) δ : 7.34-7.00 (m, 40H, aromatic),

5.84 (d, 1H, NH), 5.68 (d, $J_{1'',2''} = 4.0$ Hz, 1H, H-1''), 4.94-4.35 (m, 22H, benzyl, OCH_2O , H-1 and H-1'), 4.35 (q, $J_{5'',6''} = 6.5$ Hz, 1H, H-5''), 4.16-3.37 (m, 20H, sugar, OCH_3 (δ 3.65) and aglyconic), 2.27 (t, 2H, CH_2CO), 1.74 (s, 3H, NHCOCH_3), 1.60-1.17 (m, 15H, H-6'' (δ 1.18, $J_{5'',6''} = 6.5$ Hz) and aliphatic); ^{13}C nmr (CDCl_3) δ : 174.25 (COOCH_3), 170.12 (NHCOCH_3), 139.12, 139.07, 138.85, 138.71, 138.36, 138.22, 138.16, 138.01 (quat. aromatic), 128.96-126.58 (18 lines, remaining aromatic), 101.73 (C-1), 100.46 (C-1'), 97.73, 95.98, 95.19 (C-1'' and OCH_2O), 84.14 (C-4), 79.78, 78.46, 76.48, 76.42, 76.07, 75.34, 74.99, 74.57, 83.67, 73.49, 73.05, 72.81, 71.49, 69.86, 69.65, 69.42, 68.65, 67.00, 66.69 (sugar, aglyconic and benzylic: 21 lines expected, 19 lines found), 54.77 (C-2), 51.36 (OCH_3), 34.14 (CH_2CO), 29.76, 29.64, 29.21, 29.12, 26.00, 24.98 (aliphatic), 23.40 (NHCOCH_3), 16.82 (C-6''). Anal. calcd. for $\text{C}_{88}\text{H}_{105}\text{O}_{19}\text{N}$: C 71.38, H 7.15, N 0.95; found: C 71.61, H 7.30, N 0.95.

Evaporation of the earlier fraction (Rf 0.58) provided 8-methoxycarbonyloctyl 2-amino-4-O-[3,4,6-tri-O-benzyl-2-O-(2,3,4-tri-O-benzyl- α -L-fucopyranosyl)- β -D-galactopyranosyl]-3,6-di-O-benzyloxymethyl-2-deoxy- β -D-glucopyranoside (63) as a syrup (191 mg, 22%): $[\alpha]_{\text{D}}^{25} -24.8$ (C 1.1 CHCl_3); ^1H nmr (CDCl_3) δ : 7.34-6.99 (m, 40H, aromatic), 5.66 (d, $J_{1',2'} = 4.0$ Hz, 1H, H-1''), 4.98-4.38 (m, 21H, benzyl, OCH_2O , and H-1'), 4.25 (q, $J_{5'',6''} = 5.6$ Hz,

1H, H-5"), 4.19 (d, $J_{1,2} = 8.0$ Hz, 1H, H-1), 4.16-3.24 (m, 19H, sugar, OCH_3 (δ 3.66) and aglyconic), 2.80 (dd, $J_{1,2} = 8.0$ Hz, $J_{2,3} = 9.5$ Hz, 1H, H-2), 2.28 (t, 2H, CH_2CO), 1.70-1.11 (m, 17H, NH_2 , H-6" (δ 1.12, d, $J_{5'',6''} = 6.5$ Hz) and aliphatic); ^{13}C nmr (CDCl_3) δ : 174.13 (COOCH_3), 139.10, 138.95, 138.88, 138.69, 138.32, 138.19, 137.97 (quat. aromatic), 128.47-126.42 (15 lines, remaining aromatic), 104.31 (C-1), 101.15 (C-1'), 97.62, 97.21, 95.25 (C-1'', OCH_2O), 84.17 (C-4), 82.00, 79.63, 78.30, 75.98, 75.52, 74.84, 74.41, 73.59, 73.53, 73.37, 72.96, 72.67, 72.53, 71.26, 70.35, 69.88, 69.64, 68.73, 66.48, 66.30 (sugar, benzyl and aglyconic: 21 lines expected, 20 lines found), 56.62 (C-2), 51.31 (OCH_3), 34.08 (CH_2CO), 29.70, 29.21, 29.15, 29.08, 26.03, 24.94 (aliphatic), 16.70 (C-6").

Treatment of 63 with methanol-acetic anhydride (2:1) at room temperature overnight resulted in its quantitative conversion to 64, as evidenced by TLC, ^1H and ^{13}C nmr.

8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-[2-O-(α -L-fucopyranosyl)- β -D-galactopyranosyl]- β -D-glucopyranoside (5)

Compound 64 (323 mg, 0.22 mmol) was dissolved in 95% ethanol (8 mL) containing 5% palladium on carbon (340 mg) and hydrogenated at 140 psi for 22 h. TLC examination at this point showed the presence of a single spot (R_f 0.54 in 2-propanol-water, 8:2). The catalyst was removed by filtration on Celite and washed with several portions of hot ethanol. Solvent removal followed by freeze-drying an aqueous solution of the residue provided the title trisaccharide as a white powder (131 mg, 86%): $[\alpha]_D^{25}$ -70.4 (C 0.9 H_2O).

The 1H and ^{13}C nmr parameters of 5 appear in Tables 1 and 2, respectively.

Treatment of 5 with 85% hydrazine-hydrate, as described for the preparation of the hydrazide of 4, resulted in its complete conversion to the hydrazide as evidenced by its 1H nmr spectrum.

8-Methoxycarbonyloctyl 2-Acetamido-4-O-(3,4,6-tri-O-benzyl- β -D-glucopyranosyl)-3,6-di-O-benzyloxymethyl-2-deoxy- β -D-glucopyranoside (66)

Compound 60 (633 mg, 1.00 mmol) was engaged for 44 h in the Koenigs-Knorr reaction with 2-O-acetyl-3,4,6-tri-O-benzyl- α -D-glucopyranosyl bromide (53) [freshly prepared from 50 (810 mg, 1.56 mmol)] under the conditions described for the preparation of 54. Processing of the reaction mixture, as before, followed by chromatography, using ethylacetate-diethyl ether-n-hexane (2:2:1) as eluent, provided a fraction (Rf 0.54, 514 mg) whose $^1\text{Hnmr}$ spectrum was consistent with that of a mixture of disaccharide derivatives. De-O-acetylation, using sodium methoxide in methanol, followed by chromatography, using ethylacetate-n-hexane (2:1) as eluent, provided the title compound (Rf 0.50) as a clear syrup (331 mg, 31%): $^1\text{Hnmr}$ (CDCl_3) δ : 7.44-7.14 (m, 25H, aromatic), 5.69 (d, 1H, NH), 4.99-4.34 (m, 16H, OCH_2O , H-1' (δ 4.72, $J_{1',2'} = 8.5$ Hz), H-1 (δ 4.51, $J_{1,2} = 7.5$ Hz) and benzyl), 4.08-3.36 (m, 17H, sugar, OCH_3 (δ 3.68) and aglyconic), 3.10 (br, 1H, OH), 2.28 (t, 2H, CH_2CO), 1.75 (s, 3H, COCH_3), 1.70-1.20 (m, 12H, aliphatic).

Evaporation of the latter fraction (Rf 0.38) provided

8-methoxycarbonyloctyl 2-acetamido 4-O-(3,4,6-tri-O-benzyl- α -D-glucopyranosyl)-3,6-di-O-benzyloxymethyl-2-deoxy- β -D-glucopyranoside (65) (119 mg, 11%): $^1\text{Hnmr}$ (CDCl_3) δ : 5.12 (d, $J_{1',2'} < 3$ Hz, 1H, H-1').

8-Methoxycarbonyloctyl 2-Acetamido-4-O-[3,4,6-tri-O-benzyl-2-O-(2,3,4-tri-O-benzyl- α -L-fucopyranosyl)- β -D-glucopyranosyl]-3,6-di-O-benzyloxymethyl-2-deoxy- β -D-glucopyranoside (67)

Compound 66 (245 mg, 0.23 mmol) and the fucosyl bromide 43 (2.2 eq) were engaged in the halide-ion catalyzed reaction under the conditions described for the preparation of 64, except that a further addition of 43 (2.2 eq) was made after 15 h. After an additional 24 h, the reaction mixture was filtered, washed and evaporated to leave a dark syrup. Decolorization, using dichloromethane-ethylacetate (1:1), followed by chromatography, eluting with dichloromethane-ethylacetate (4:1), provided the title compound (Rf 0.38) as a clear syrup (193 mg, 57%): $[\alpha]_D^{25} -13.0$ (C 0.2 CHCl₃); ¹Hnmr (CDCl₃) δ : 7.45-7.04 (m, 40H, aromatic), 5.74 (d, 1H, NH), 5.57 (d, J_{1'',2''} = 3.5 Hz, 1H, H-1''), 4.98-4.42 (m, 22H, benzyl, OCH₂O, H-1 and H-1'), 4.30 (q, J_{5'',6''} = 6.5 Hz, 1H, H-5''), 4.08-3.34 (m, 20H, sugar, OCH₃ (δ 3.67) and aglyconic), 2.28 (t, 2H, CH₂CO), 1.79 (s, 3H, COCH₃), 1.70-1.19 (m, 15H, H-6'' (δ 1.20, J_{5'',6''} = 6.5 Hz) and aliphatic); ¹³Cnmr (CDCl₃) δ : 174.16 (COOCH₃), 170.10 (NHCOCH₃), 138.92, 138.86, 138.57, 138.42, 138.32, 138.23, 137.99 (quat. aromatic), 128.48-126.54 (16 lines, remaining aromatic), 101.30 (C-1), 100.50 (C-1'), 97.99, 96.23, 95.24 (C-1'', OCH₂O), 85.63 (C-4),

79.91, 78.48, 78.38, 76.65, 76.31, 75.98, 75.17, 75.07,
75.01, 74.77, 74.32, 73.68, 73.51, 72.89, 69.71, 69.42,
68.95, 66.83, 66.52 (sugar, benzyl and aglyconic: 21 lines
expected, 19 lines found), 55.73 (C-2), 51.32 (OCH₃), 34.13
(CH₂CO), 29.64, 29.18, 29.09, 25.97, 24.97 (aliphatic),
23.44 (NHCOCH₃), 16.80 (C-6").

Anal. calcd. for C₈₈H₁₀₅O₁₉N: C 71.38, H 7.15, N 0.95;
found: C 71.22, H 7.27, N 1.04.

8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-4-O-[2-O-(α -
L-fucopyranosyl)- β -D-glucopyranosyl]- β -D-glucopyranoside (6)

Compound 67 (162 mg, 0.109 mmol) was hydrogenated for 48 h under the conditions described for the preparation of 5. The yield of white freeze-dried powder (Rf 0.60 in 2-propanol-water, 8:2) was 65 mg (85%): $[\alpha]_D^{25} -78.7$ (C 0.5 H₂O).

The ¹H and ¹³Cnmr parameters are reported in Tables 1 and 2, respectively.

Treatment of 6 with hydrazine-hydrate, as described for the preparation of the hydrazide of 5, resulted in its complete conversion to the hydrazide as evidenced by ¹Hnmr spectroscopy.

B. Immunochemistry

1. Preparation of Antigens and Immunoadsorbents.

The procedure used for the preparation of the BSA-antigens was slightly modified from that reported by Lemieux, Bundle and Baker.¹¹ The hydrazine-free hydrazides of 2, 4, 5 and 6 were obtained as white powders by filtration through a column of Biogel P-2 in 10% aqueous ethanol followed by evaporation and freeze-drying of an aqueous solution of the eluate. The hydrazine-free hydrazide (100 μ mol) was dissolved in dry DMF (1.5 mL) and cooled to -25°C . Then 400 μ mol of 3.6M HCl in anhydrous dioxane were added, followed by t-butyl nitrite (140 μ mol). After 30 min, sulphamic acid (40 μ mol) in DMF (100 μ L) was added and the solution kept at -25°C for an additional 15 min. This solution containing the hapten azide was added to a solution of BSA (2 μ mol) in 23 mL of buffer solution (0.085M HCl in 0.2M N-ethyl diethanolamine; pH 8.9) and kept at $0 - 4^{\circ}\text{C}$ for 12 h and at room temperature for 2 h. This solution was dialyzed against doubly distilled water (300 mL) in a Diaflo ultrafiltration cell equipped with a PM-10 membrane and freeze-dried to provide the antigen as a white powder.

The antigens were analyzed for carbohydrate content by the phenol-sulfuric acid method⁹⁸ using a standard curve obtained from a series of solutions containing known

concentrations of the free haptens. The hapten incorporations achieved are reported as the number of moles of bound hapten per mole of antigen, on the basis of a molecular weight for BSA of 65,000,⁹⁷ in Table 7.

For the preparation of an immunoabsorbent (IA), the hapten acyl-azide was prepared in the same manner as described above except that Hunig's base (500 μ mol) was used in place of the sulphamic solution. The solution containing the hapten-azide was then added to a slurry of silyl-aminated Chromosorb P⁹⁶ suspended in acetonitrile (550 mL) and kept stirring at 0 - 4°C for 12 h and room temperature for 2 h. The IA was collected on a scintered-glass funnel and washed thoroughly with methanol and diethyl ether, and then stirred with 10% acetic anhydride in methanol at room temperature for nearly 12 h. The IA was then washed exhaustively with methanol, water, saturated sodium hydrogen carbonate solution, water, methanol and ether and air dried. The hapten-incorporations achieved were determined as described for the antigens and are reported as the number of μ moles of hapten per gram of IA in Table 7.

2. Immunizations.

San Juan rabbits were immunized, in groups of 3, with each of the antigens derived from 1 - 6. The immunization

Incorporation

<u>Hapten</u>	<u>Antigen (mol/mol)</u>	<u>Immunoabsorbent (μmol/g)</u>
e-Le ^a (2) _~	14.2	0.40
e-Le ^d (4) _~	18.5	0.50
H (5) _~	13.8	0.55
e-H (6) _~	19.1	0.37

TABLE 7. Incorporation of hapten into the BSA-antigen and immunoabsorbent estimated by the phenol-sulfuric acid method.⁹⁸

schedule used was that described by Lemieux et al.¹¹ as Protocol A. The antigen (0.5 mg) was administered in phosphate-buffered saline (1.6×10^{-4} M NaH_2PO_4 and 5×10^{-4} M Na_2HPO_4 in 0.15M NaCl; PBS) emulsified in Freund's complete adjuvant (FCA) (0.8 mL). The suspension (0.2 mL) was injected subcutaneously in each toe pad and in the neck. The remaining 0.4 mL was injected intramuscularly, 0.2 mL into each rear thigh. A further 1 mg of antigen in PBS (1.5 mL) and FCA (1.4 mL) was given over 5 separate injections 3 or 4 days apart, 0.3 mL in each rear thigh muscle. The rabbits were exsanguinated by cardiac puncture 10-12 days after the last injection. The blood was collected in vacutainer tubes and incubated at 37°C for 1 h and 0 - 4°C for 1 - 2 h prior to clot retraction. The sera were made 0.5% in sodium azide and stored at 0 - 4°C.

3. Quantitative Batch Immunoabsorption (QBIA)¹⁰¹

The Le^a , Le^d , Le^c , Le-disac, GlcNAc, LacNAc and H-disac IA's were available in these laboratories. The antibody binding capacity of all the IA's used was estimated by adsorption of the undiluted anti-serum obtained from a rabbit immunized with the BSA-antigen derived from the same hapten. In the few cases where the result of the QBIA experiment indicated that the IA was within 20% of saturation using 100 μL of serum, the experiment was repeated using 50 μL and this result was used.

In a typical experiment, the serum (100 μ L) was added to a 12 x 75 mm test-tube containing the IA (30 ± 1 mg) and PBS (900 μ L) and the tube was slowly rotated, at an angle of about 45° , at $0 - 4^\circ\text{C}$ for 2.5 h. The supernatant was removed and the IA was washed 6 times with PBS (4 mL). Residual PBS was removed by suction with a finely-drawn capillary. Then 2% NH_4OH (in 0.15 NaCl) (1.0 mL) was added and, after rotation of the tube for 10 min at $0 - 4^\circ\text{C}$ and centrifugation at 3000 rpm (1000 g) for 2 min, the supernatant was removed. The optical density of this solution at 280 nm was determined using the corresponding solution obtained by identical treatment of a blank (un-haptenated) IA as standard. The antibody concentration of the serum was then calculated using the extinction co-efficient $E_{280}^{1\%} = 14,^{101}$ according to the expression:

$$\text{antibody concentration (mg/mL)} = \frac{A_{280} \text{ (obs)}}{\text{mL of serum (usually 0.1)} \times 1.4}$$

The results of the 96 QBIA experiments performed are presented in Tables 4 and 5.

4. Isolation of Antibodies.

The general procedure for the isolation of antibodies from animal serum is given below. All manipulations were

carried out at 0 - 4°C.

The serum (10 mL) was passed through a column (1.5 cm I.D.) of IA (10 g) at a flow rate of 20 mL/h and the column was washed with PBS until no U.V. (280 nm) adsorption was detectable in the eluate. Then 2% NH_4OH (in 0.15M NaCl) was passed through the column at a flow rate of 140 mL/h and the protein containing eluate was collected and immediately neutralized with KH_2PO_4 to a pH of ca. 7.2. This solution was dialyzed (XM-50 membrane) exhaustively against PBS, concentrated to 10 mL and sterilized by millipore filtration. QBIA assays showed, in all cases, that about 80% of the desorbed protein was active-antibody.

5. N-Deacetylations.

All the N-deacetylations were performed under conditions modified from those described by Lindberg et al.¹¹⁵ The haptens (N-acetylated methyl esters) (ca. 5 mg) were dissolved in dimethylsulfoxide (2.0 mL) and 10N sodium hydroxide (0.4 mL). Thisphenol (50 μL) was added and the solutions were heated in a stainless steel bomb at 120°C for hearly 65 h. After cooling, the reactions were diluted with water (10 mL) and neutralized with acetic acid. Evaporation to dryness left a gummy white solid which was extracted 4 times with diethyl ether (25 mL). This material was dissolved in 10% ethanol and filtered through 2 columns

(1.5 x 30 cm) of Biogel P-2, using the same solvent, to remove the sodium acetate. Evaporation of the eluate and freeze-drying of the residue provided the N-deacetylated carboxylic acids as white powders. The ^1H nmr spectra of these samples, recorded in D_2O , were devoid of signals for the methoxyl and acetamido methyl group protons of the starting materials. These spectra were all in agreement with the assigned structures and required high purity. All the free amines provided the signal for H-2 as a doublet of doublets between 2.9 and 3.1 ppm.

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APPENDIX

SPIRO-1,2-ACYLORTHOESTERS OF 3,4,6-TRI-
O-ACETYL- α -D-GLUCOPYRANOSE

RAYMOND U. LEMIEUX and OLE HINDSGAUL

ABSTRACT

The 1,2-O-(2 - oxa-3 - oxo-cyclopentylidene) derivative of 3,4,6-tri-O-acetyl- α -D-glucopyranose was prepared in both the exo (4) and endo (5) forms. The compounds were prepared by bromide-ion promoted cyclization of 3,4,6-tri-O-acetyl-2-O-(3-carboxypropionyl)- α -D-glucopyranosyl bromide. The similar acylorthoester derivatives of phthalic acid were prepared from 3,4,6-tri-O-acetyl-2-O-(2-carboxybenzoyl)- α -D-glucopyranosyl bromide. The cyclizations produced a much higher ratio of the endo forms than would be expected from their relative thermodynamic stabilities. The configurations were established by nuclear Overhauser enhancement studies and their conformations deduced from ^1Hmr parameters. The greater stability of the exo-isomers appears to have a stereoelectronic origin. Preliminary efforts to engage the acylorthoesters in reactions with isopropyl alcohol to form glycosides are reported. It was discovered that carboxylic acid provides powerful catalysis for the β to α anomerization of O-acylated glucopyranosides by stannic chloride.

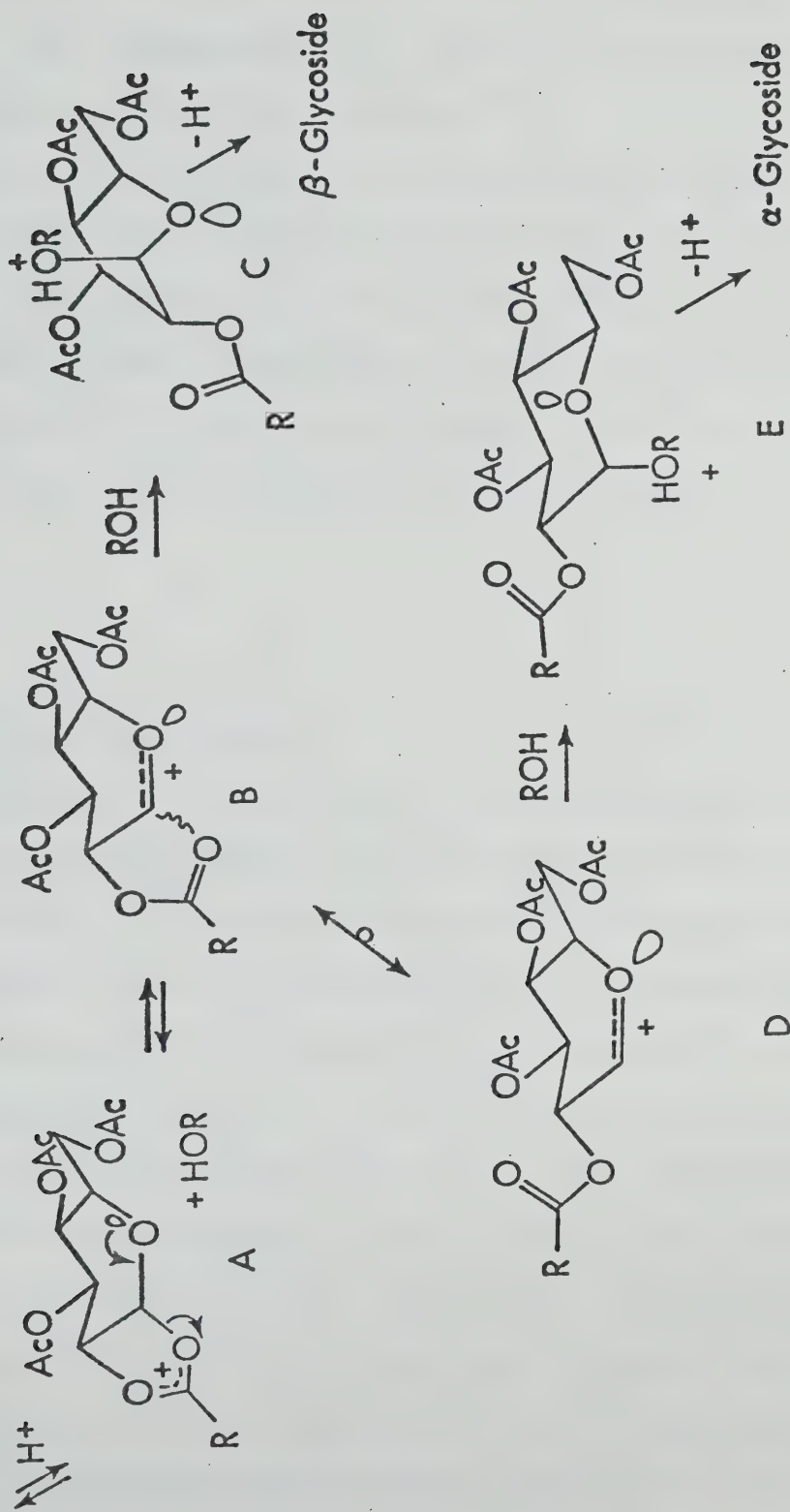
INTRODUCTION

The development of a generally reliable synthesis of 1,2-trans- β -glycopyranosides in high yield remains an important challenge. The best methods now available, using

glucose derivatives as an example, appear to be those involving the intermediate formation of a 1,2-orthoester.^{1,2} All such methods, especially when applied to hindered and weakly nucleophilic alcohols, are susceptible to extensive formation of α - as well as β -glycoside.^{3,4,5} Also, the nature of the acid used to catalyze the orthoester rearrangement can strongly influence the route of reaction.^{3,6}

The formation of a β -glycoside by way of a 1,2-orthoester is expected to proceed by the reaction pathway outlined in Scheme 1. The first formed 1,2-dioxenium ion (A) is attacked at the anomeric rather than the 2-position of the pyranose ring because of participation of the O-5 atom in charge delocalization as indicated. Most probably, the reaction, in its first stage, involves bond rearrangement of the dioxenium ion (A) toward the glycosyloxonium ion (B). Nucleophilic attack at the β -side of the anomeric center would be rendered favorable because of shielding of the α -side by the newly formed 2-acetoxy group which can be expected to be electrostatically attracted to the carbonium center as depicted in B. Stereoelectronic demands appear⁷ to require the development of the glycosidic bond to occur with a p-orbital of the O-5 atom in an anti-periplanar orientation as indicated in C. Should the shielding of

Orthoester



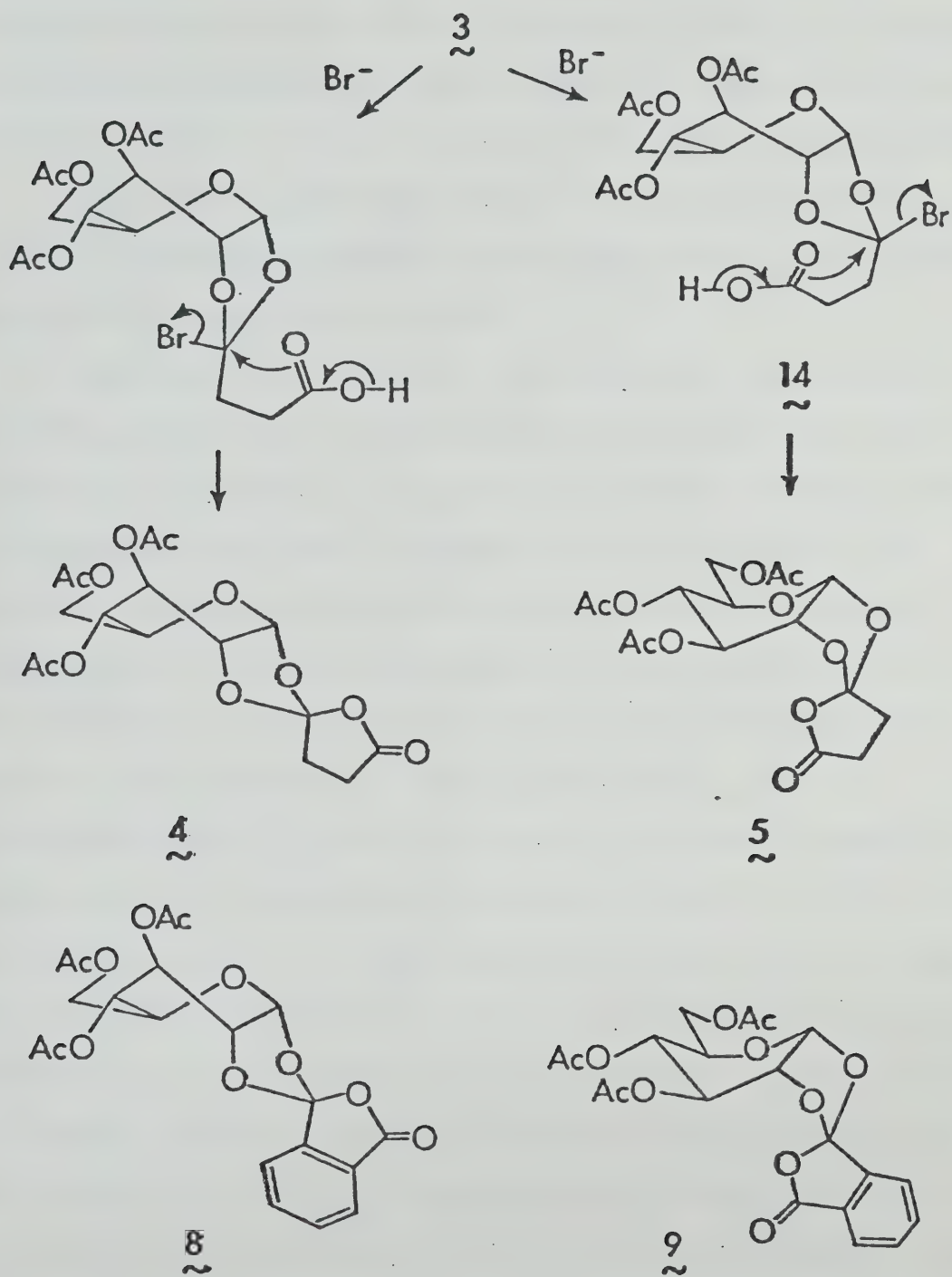
Scheme 1 Reactive intermediates postulated for the acid-catalyzed alcoholysis of a 1,2-orthoester to O-acetylated anomeric glucopyranosides.

the α -side of the anomeric center be lost, as is indicated in D, then α -glycoside (E) formation is expected to become the favored route of reaction .⁷

It was considered of interest in this regard to prepare intramolecular 1,2-acylorthoesters such as could be derived from succinic acid (4 and 5) and phthalic acid (8 and 9) to inhibit the accumulation of stable orthoesters derived from external alcohol and, perhaps, favor reaction by way of the oxocarbonium ion of type B rather than type D.

DISCUSSION OF RESULTS

3,4,6-Tri-O-acetyl-2-O-(3-carboxypropionyl) α -D-glucopyranosyl bromide (3) was prepared from the readily available 1,3,4,6-tetra-O-acetyl- α -D-glucopyranose (1) by way of its 2-O-(3-carboxypropionyl) derivative (2). The intramolecular cyclization of 3 to produce the desired spiro acylorthoesters (4 and 5) in excellent yield was induced by bromide-ion catalysis.⁸ The corresponding derivatives of phthalic acid (8 and 9) were prepared in a similar manner from 7. Fractional crystallization allowed the isolation of the pure orthosuccinate 5 and the orthophthalate (8). Attempts to obtain pure samples of 4 and 9 by chromatographic separation failed. The properties of these compounds were deduced from mixtures of the epimers.



The ^1H and ^{13}C mr parameters determined for compounds 4, 5, 8 and 9 are reported in Table I together with those for the structurally related compounds 10 to 13. The exo- and endo-configurations were assigned to compounds 4 and 5 on the basis of nuclear Overhauser enhancement measurements.

The readily available form of 3,4,6-tri-O-acetyl-1,2-O-(1-ethoxyethylidene)- α -D-glucopyranose (10) has been assigned the exo-configuration.⁸

The great stability and uniformity of the magnetic field generated by a superconducting solenoid, the high resolution achieved at fields up to 400 MHz together with the computer-assisted FT mode of operation allows the precise (within 2%) measurements of nuclear Overhauser enhancements⁹ and thereby often provides a convenient tool for estimating whether or not two or more hydrogen atoms are in close proximity. Thus, for example, the exo-configuration of 10 could be easily confirmed by examining the effect of irradiating the methyl group (δ : 1.70) of the orthoacetyl unit. As expected, the signals for H_3 , H_5 and the methylene hydrogens of the ethoxy group were uniquely enhanced by 2.4, 9.5 and 5.6%, respectively. This places the methyl group closer to H-5 than H-3 which would be in accordance with the $\text{B}_{2,5}$ conformation (however, as will be seen below, in a somewhat distorted form). Similarly, irradiation of the higher field (δ 2.65) multiplet for the methylene protons of the succinyl residue of 4 resulted in 2.4 and

TABLE I. Nuclear Magnetic Resonance Parameters^a

Compound	H-1	J _{1,2}	H-2	J _{2,3}	H-3	J _{3,4}	H-4	J _{4,5}	C-1	C-2	C-3	C-4	C-5	C-6	C ^b	Orthoester Residue
Exo-epimers^c																
4	5.82	5.5	4.44	3.0	5.18	3.0	4.92	9.5	97.32	73.52	69.68	67.98	67.43	62.99	126.47	-OCH ₂ -CH ₂ -C(=O)- (2.65(m), 2.80(m))
8	6.04	5.5	4.68	3.2	5.32	3.2	5.03	8.5	97.74	74.12	69.97	67.92	67.92	63.17	122.76	-----
10	5.71	5.5	4.31	2.9	5.17	2.9	4.90	9.5	97.06	73.39	70.42	68.45	67.18	63.22	121.41	CH ₃ -OCH ₂ -CH ₃ 1.70(s), 3.54(q), 1.17 (t)
11 ^d	5.68	5.0	4.20	3.0	5.18	3.0	4.89	8.5	96.66	73.47	70.88	68.55	67.00	63.22	109.83	-----
12 ^e	5.69	5.0	4.35	3.0	5.19	3.0	4.91	9.0								-----
Endo-epimers																
5	5.80	6.2	4.40	4.5	5.46	7.2	5.01	9.7	99.20	76.74	73.22	67.25	68.48	61.95	127.27	-OCH ₂ -CH ₂ -C(=O)- 2.48(m), 2.75(m)
9	6.07	6.0	4.65	4.5	5.63	7.5	5.11	10.0	99.93	77.23	73.33	67.30	68.70	62.00	122.93	-----
13 ^e	5.61	5.5	4.25	4.7	5.56	7.0	4.99	9.5								-----

^aAll spectra were obtained in CDCl₃. Chemical shifts (δ) are reported in ppm relative to internal TMS. First order coupling constants are in Hz for spectra measured at 100 MHz.

^bQuaternary carbon.

^cAll these isomers showed long-range coupling between H-2 and H-4 (1.0 Hz) as found by Coxon and Hall¹¹ for 1,2-O-alkylidene derivatives of glucose. The skew B_{2,5} conformation is compatible (W arrangement) with this coupling.

^d3,4,6-Tri-O-acetyl-1,2-O-isopropylidene-α-D-glucopyranose.¹⁸

^eThe exo (12) and endo (13) isomers for 3,4,6-tri-O-acetyl-1,2-O-(2-oxacyclopentylidene)-α-D-glucopyranose.¹⁸

6.4% enhancements of the signals for H₃ and H₅ respectively. Alternately, irradiation of H-5 of 4 caused a 2.1% enhancement of the signal for this methylene group. As expected, the signals for H-3 (6%) and the two H-6's (4.7%) were also enhanced. These measurements allow unequivocal assignment of the configuration of the quaternary carbon in 4; that is, 4 is the exo-isomer. In the case of 5, irradiation of the multiplet at $\delta = 2.48$ caused enhancement of the signals for H-1 (4.0%) and H-2 (2.3%) as expected for the endo-isomer. The conformational preferences for 4 and 5 are discussed in detail below. The configurations of the phthalic acid derivatives (8 and 9) are assigned on the basis of the nmr parameters reported in Table I when compared to those for compounds 4, 5 and 10.

Under the conditions for the bromide-ion catalyzed cyclizations of 3 and 7 the ratio of exo to endo acyl-orthoester found in the product varied somewhat from experiment to experiment but in every case the amount of the exo product was less than that in mixtures obtained when the isomers were equilibrated in dichloromethane containing a trace of trifluoroacetic acid. Exo-4 and endo-5 were formed in a ratio of near 3:5 whereas the ratio was 5 for the equilibrium mixture. In the case of exo-8 and endo-9, the ratio in the reaction product was 3:2 whereas in the equilibrium mixture the ratio was 7:2. In the case of the ethyl orthoacetate 10, the ratio of exo to endo in the equilibrium mixture was the same

as that found for 4 and 5. The rapid equilibrations are expected to proceed by way of 1,2-dioxenium ions of the type 15 with A = H. The faster formation of the thermodynamically less stable endo-5 from 3 can be rationalized in terms of an S_N2-type replacement, as depicted by 14, of exo-bromide formed from 15 (A = H) and bromide ion.

From the standpoint of repulsive non-bonded interactions, it could be expected that the more stable acylorthoester would be that with the "small" oxygen atom in the more crowded endo position. However, as noted above, it is the exo-isomer that is energetically more favorable.

The modified Karplus relationship¹⁰ used by Coxon and Hall¹¹ in a study of the conformations of 1,2-O-alkylidene pyranose derivatives provides the torsion angles given in Table II for the vicinal hydrogens about the pyranose rings of the orthosuccinates 4 and 5.

The difference in the coupling constants for vicinal hydrogens about the pyranose rings of the orthosuccinates 4 and 5 requires the change in configuration at C-2 of the dioxolane ring to cause an appreciable change in the conformation of the pyranose ring. As seen above, the nuclear Overhauser enhancement experiment favors a B_{2,5}-like conformation for 4. Indeed, inspection of a molecular model suggests that 4 likely exists in the somewhat flattened and slightly distorted B_{2,5}-conformation which is inferred by the vicinal coupling constants (Table II). In the case

TABLE II

Conformations for the Spiro-1,2-acylorthoesters
 Estimated^a from Vicinal Coupling Constants^b

	<u>H-1,H-2</u>	<u>H-2,H-3</u>	<u>H-3,H-4</u>	<u>H-4,H-5</u>
Compound 4 (exo)				
$^3J_{H,H}$ (Hz)	5.5	3.0	3.0	9.5
Torsion angle (°)	-38	-54	124	166
Compound 5 (endo)				
$^3J_{H,H}$ (Hz)	6.2	4.5	7.2	9.7
Torsion angle (°)	±33	-133	148	169

^aCalculated using the expression proposed
 by Abraham and coworkers¹⁰.

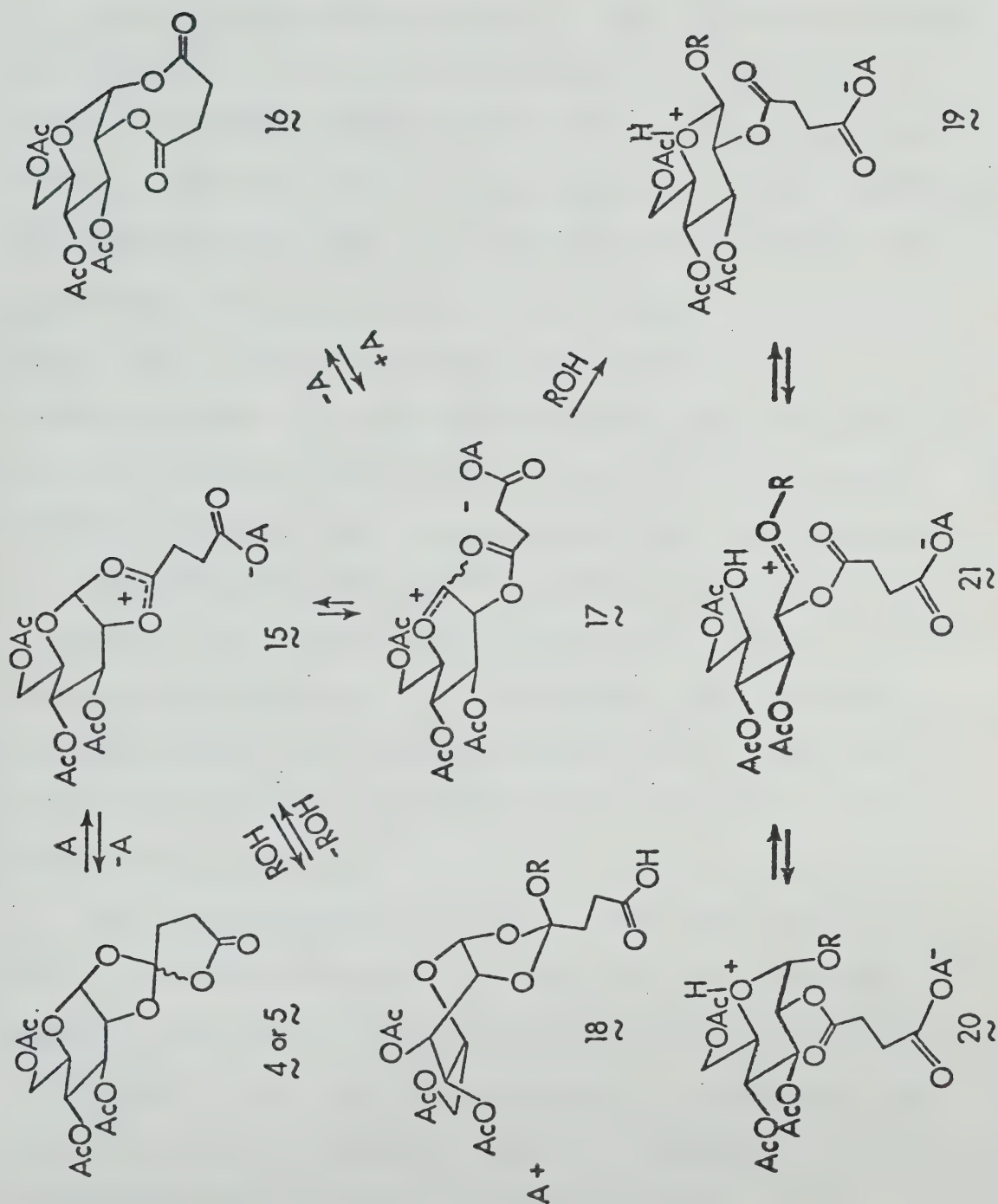
^bAs derived from the observed spacings.

of 5, the large values for $J_{H-3,H-4}$ and $J_{H-4,H-5}$ are suggestive of near anti-periplanar orientations between H-4 and its neighboring hydrogens (H-3 and H-5). The value of 6.2 Hz for $J_{H-1,H-2}$ requires these hydrogens to define a torsion angle of about 30° . On this basis, the preferred conformation for the pyranose ring of 5 could be near the ${}^{1,4}B$ conformation with H-1 and H-2 in quasi-axial and quasi-equatorial orientation, respectively. On this basis, it would be surprising that irradiation of the methylene group would cause stronger enhancement of the quasi-axial H-1 atom. On the other hand, the 4C_1 conformation for the pyranose ring which has the O-5-C-1-C-2 region strongly flattened so as to accommodate the dioxolane ring also provides torsion angles compatible with the measured vicinal coupling constants (Table II) but a decision in this regard could not be made on the basis of the coupling constants only.

In order to rationalize the fact that the exo-compound 4 is thermodynamically more stable than its endo-isomer 5, it seems necessary to invoke a substantial driving force for the oxygen atom at the 2-position of the dioxolane ring to adopt as axial an orientation as possible as indicated by the conformational formulas presented for 4 and 5 (also, the related structures 8 and 9). The driving force presumably would be of a stereoelectronic origin as is the case for the anomeric effect¹². On this basis, a rationalization seems possible

since, should the $B_{2,5}$ conformation of 4 be maintained in the case of 5, there would exist, as can be readily gauged from a molecular model, a severe non-bonded interaction between the endo-oxygen atom and H-5. Thus, the change in conformation for the pyranose ring would be expected to occur in order to relieve this interaction. Should the endo-isomer (5) prefer the flattened 4C_1 -like conformation then, as suggested by the conformational drawing, the endo-oxygen atom would be in a position to cause deshielding of H-3 of 5 as compared to H-3 of 4. As seen in Table I, this was indeed the case. Furthermore, this conformation would place H-1 in a somewhat more equatorial orientation than H-2 and these orientations would be more in line with the nuclear Overhauser enhancement which were observed on the irradiation of the methylene-group hydrogen atoms of 5 than would be the case should the pyranose ring be in the ${}^{1,4}B$ conformation. It must be recognized that the conformations assigned to the pyranose rings of the acylorthoesters represent the weighted average of the conformers with appreciable population in the conformational equilibria and, likely, these are not highly rigid structures.

It was expected that acylorthoesters would be highly prone to dissociation either by thermal excitation alone or with the assistance of acid catalysis to form dipolar species (15) when A is a neutral species (solvent or Lewis acid) or the cationic species when A is a proton. Recently, Wulff and co-workers¹³ reported the synthesis



and reactivity of 3,4,6-tri-O-acetyl-1,2-O-(1-[4-(4-biphenyl)butyryloxy]ethylidene)- α -D-glucopyranose.

In principle, the dioxenium ion (15) could lead to the formation of the cyclic structure 16. However, this structure was not encountered either in the preparation of the acylorthoesters or in the product of their solvolysis. It can be expected that 15 would be in equilibrium with 17 so that, in the presence of an alcohol, a glycoside would form. Under conditions which would produce the dipolar species 17, the α -side could be well shielded and thus promote attack by the alcohol on the β -side to form β -glycoside as the preferred route of reaction.

Reaction of 4 and 5 with alcohol in the presence of a trace of trifluoromethanesulfonic acid as catalyst could lead to the formation of the orthoester 18. However, a two mole excess of isopropyl alcohol did not provide a measurable amount of 18 under conditions which rapidly equilibrate 4 and 5.

The acylorthoesters (4 and 5) were then heated at 150° in nitrobenzene with a molar excess of isopropyl alcohol. Only 60% of the orthoesters reacted in 18 h. An isopropyl ester was formed along with glycoside (30% yield) in a β to α ratio of 5 to 1. Addition of 2,4,6-trimethylbenzoic acid had no apparent affect on the reaction. Addition of a slight excess of 2,6-lutidine completely suppressed the formation of glycoside. Judging

from these results, the further pursuit of this approach to the synthesis of β -glycosides should include a search for more reactive spiro-acylorthoesters.

Stannic chloride is known to promote the reaction of an acetylated sugar with alcohols to form glycosides.^{14,15} It was therefore of interest to examine the reaction of these acylorthoesters promoted by this Lewis acid. Indeed, reaction of 5 was rapid at 25°, with dichloromethane as solvent, providing near quantitative yields of isopropyl glycoside but with the α -anomer predominating. Examination of the course of reaction showed the system to provide strong conditions for β to α glycoside anomerization. This was surprising since Pacsu¹⁶ had shown stannic chloride to be a very poor catalyst for this transformation and this was recently confirmed.¹⁵ Indeed, isopropyl 2,3,4,6-tetra-O-acetyl β -D-glucopyranoside (22) (0.1 M) in chloroform at 25° and in the presence of an equimolar amount of stannic chloride was found to anomerize very slowly (half time of reaction about 1400 min). However, under the same conditions, isopropyl 3,4,6-tri-O-acetyl-2-O-(3-carboxypropionyl)- β -D-glucopyranoside (24) and isopropyl 3,4,6-tri-O-acetyl-2-O-(2-carboxybenzoyl)- β -D-glucopyranoside (25) were found to undergo rapid anomerization with half-lives of 10 and 7 minutes, respectively.

These rapid rates of reaction were expected to originate in the liberation of a proton to form the protonated species 19 and 20 ($A=\text{SnCl}_4$) under superacid conditions, with subsequent anomerization, probably via the open chair intermediate (21)¹⁷. Indeed, when isopropyl 2,3,4,6-tetra-O-acetyl- β -D-glucopyranoside (22) was subjected to the above conditions, with the addition of one molar equivalent of acetic acid, anomerization was near 100 times faster ($t_{1/2} = 14$ min) than in the absence of the acetic acid.

In contrast to the results obtained using the succinyl compounds 4 and 5, the reaction of the phthalyl compounds 8 and 9 produced high yields of 3,4,6-tri-O-acetyl-2-O-(2-carboxybenzoyl)- α -D-glucopyranosyl chloride when treated with stannic chloride in methylene chloride in the presence of two mole equivalents of isopropyl alcohol. It thus appears important to choose structures for the development of this approach to β -glycoside synthesis that can well separate the $-\text{COOSnCl}_4^-$ group from the anomeric center.

EXPERIMENTAL

The general procedures and analytical methods used were the same as previously described.⁷

1,3,4,6-Tetra-O-acetyl-2-O-(3-carboxypropionyl)- α -D-glucopyranose (2)

A solution of 1,3,4,6-tetra-O-acetyl- α -D-glucopyranose (1)¹⁹ (6.76 g, 19.4 mmol), succinic anhydride (5.77 g, 57.6 mmol) and 4-dimethylamino pyridine (0.2 g) in 40 mL dichloromethane-pyridine (1:1, V/V) was stirred at room temperature for 2 h by which time tlc on silica gel using ethyl acetate, hexane, ethanol (10:10:1) indicated completion of reaction. Water (20 mL) was added and the solution was evaporated to dryness. Toluene (2 x 100 mL) was added to the residue and removed by distillation in vacuo.

The residue was dissolved in dichloromethane (100 mL) and the solution was washed successively with water (2 x 100 mL), 5% HCl (200 mL) and water (100 mL). Drying and solvent removal provided a syrupy product in 98% yield (8.58 g) which crystallized from ethyl acetate-hexane: mp 116-117°, $[\alpha]_D^{25} +93.4$ (C 1.01 CHCl₃).

Anal. calc. for C₁₈O₁₃H₂₄: C, 48.22; H, 5.39.

Found: C, 47.96; H, 5.44.

3,4,6-Tri-O-acetyl-1,2-O-[2 - oxa-3 - oxo-cyclopentylidene]
 α -D-glucopyranose [4 (exo), 5 (endo)]

A saturated solution of HBr in acetic acid (40 mL, prepared at 0°) containing 3% (V/V) of acetic anhydride was added to a solution of 2 (8.50 g, 19.0 mmol) in dichloromethane (10 mL). After the resulting solution had been kept at room temperature for 1.5 h, the solvent was removed at a bath temperature of 35°. Toluene (100 mL) was added to the residual yellow syrup and removed by distillation in vacuo. After repeating this treatment a second time, the product was dissolved in dichloromethane and the resulting solution was decolorized with charcoal, and evaporated to provide 3,4,6-tri-O-acetyl-2-O-(3-carboxypropionyl)- α -D-glucopyranosyl bromide (3) as a white foam. The crude product resisted crystallization but appeared essentially pure (nmr) and was used directly to form the title compounds. The material was dissolved in dry acetonitrile (30 mL) and to this solution 4 Å molecular sieve (5 g), tetraethylammonium bromide (3.2 g) and 2,6-lutidine (10 mL) were added and the mixture was stirred at room temperature for 3 h. Removal of the solids and evaporation left a yellow syrup which was dissolved in dichloromethane (150 mL). This solution was washed twice with water, dried and evaporated to leave a syrup which crystallized from dichloromethane-di-isopropyl ether as fluffy white needles, 4.43 g (60%), mp 125-135°.

The ^1Hmr of this material showed it to consist of a 5:3 ratio of 5 and 4. Successive recrystallizations from ethyl acetate and dichloromethane-diethyl ether provides pure endo isomer (5): mp 162-164°, $[\alpha]_D^{21} +135.6$ (c 1.0 CHCl_3).

Anal. Calc. for $\text{C}_{16}\text{H}_{20}\text{O}_{11}$; C, 49.49; H, 5.19.

Found: C, 49.32; H, 5.16.

1,3,4,6-Tetra-O-acetyl-2-O-(2-carboxybenzoyl)- α -D-glucopyranose (6).

A solution of 1 (11.66 g, 33.8 mmol), phthalic anhydride (7.49 g, 1.5 eq) and 4-dimethylaminopyridine (37 mg) in 45 mL pyridine-dichloromethane (2:1 V/V) was kept at room temperature for 9 h, then taken to dryness. The residual clear syrup was dissolved in dichloromethane (100 mL) and the solution was washed with 5% HCl (200 mL), then water (100 mL). Solvent removal left a white solid which crystallized from boiling ethanol (100 mL) in 90% yield (15.04 g). One recrystallization provided the analytical sample; mp 159-160°, $[\alpha]_D^{21} +107^\circ$ (c 0.99, CHCl_3).

Anal. Calcd. for $\text{C}_{22}\text{O}_{13}\text{H}_{24}$: C, 53.23; H, 4.87.

Found: C, 52.98, H. 4.84.

3,4,6-Tri-O-acetyl-2-O-(2-carboxybenzoyl)- α -D-glucopyranosyl
bromide (7).

Compound 6 (20.07 g, 40.47 mmol) was reacted with hydrogen bromide under the same conditions used for the preparation of 3, producing 7 as a white foam which provided an unstable crystalline product from ethanol-hexane: mp 140° (dec), $[\alpha]_D^{24} +179.3^\circ$ (c 1.0 CHCl₃).

Anal. Calc. for C₂₀H₂₁O₁₁Br: C, 46.44; H, 4.09; Br, 15.45. Found: C, 46.07; H, 3.98; Br, 15.70.

3,4,6-Tri-O-acetyl-1,2-O-phthalidylidene- α -D-glucopyranose
[8 (exo), 9 (endo)].

The crude 7 appeared essentially pure on examination by tlc [silica gel-ethyl acetate, hexane, acetic acid (5:5:1)] and was used directly for the preparation of the title compounds, as described for 4 and 5, except that the reaction mixture was stirred at 50° for 2 h, to give a yellow foam (90% yield).

The ¹Hmr spectrum showed it to consist of an approximately 2:3 mixture of the endo 9 and exo 8 isomers. Crystallization from methanol provided 10.4 g of pure 8 (59%): mp 150.5 - 152°, $[\alpha]_D^{24} +84.3^\circ$ (c 1.0 CHCl₃).

Anal. Calc. for C₂₀H₂₀O₁₁: C, 55.05; H, 4.62. Found: C, 55.03; H, 4.59.

Isopropyl 3,4,6-Tri-O-acetyl-2-O-(3-carboxypropionyl)-
β-D-glucopyranoside (24).

Isopropyl 3,4,6-tri-O-acetyl-β-D-glucopyranoside (23)²⁰ (800 mg, 2.3 mmol) was reacted with succinic anhydride, as described for the preparation of 2, to provide a pale yellow solid: 1.03 g (99%). Crystallization from ethylacetate-hexane provided the pure material: mp 116-117°, $[\alpha]_D^{25} +93.3^\circ$ (c 1.0 CHCl₃).

Anal. calc. for C₁₉H₂₈O₁₂: C, 50.89; H, 6.29.

Found: C, 50.82; H, 6.31.

Isopropyl 3,4,6-Tri-O-acetyl-2-O-(2-carboxybenzoyl)-β-D-
glucopyranoside (25)

Compound 23 (435 mg, 1.25 mmol) was reacted with phthalic anhydride, under the conditions described for the preparation of 6, but for 36 h. The product crystallized from ethylacetate-n-hexane (68% yield): mp: 120-121°, $[\alpha]_D^{25} -9.87$ (c 0.75 CHCl₃).

Anal. calc. for C₂₃O₁₂H₂₈: C, 55.64; H, 5.68. Found: C, 55.78; H, 5.71.

Glycosidation Reactions

1. Solvolysis reactions.

A solution of compound 5 (134 mg, 0.35 mmol) and isopropyl alcohol (0.70 mmol) in dry nitrobenzene (1.0 mL) was heated in a sealed tube at 150° for 18 h. Solvent

evaporation (100°, high vacuum) left a dark yellow syrup whose ^1Hmr (CDCl_3) showed a 60% incorporation of the isopropyl group into the non-volatile product and near 40% of equilibrated starting material (4 and 5). This material was de-O-acetylated by treatment with methanol-triethylamine-water (2:1:1) overnight at room temperature. Evaporation and treatment of a methanolic solution of the product with Amberlite IR-120(H^+) gave a mixture of glucose and isopropyl glucopyranoside (30% yield) in a ratio of approximately 7:3 (^1Hmr). The relative intensities of the signals for these anomeric protons required the isopropyl β - and α -D-glucopyranosides to be present in a ratio of 5.

2. Stannic Chloride Reactions.

Stannic chloride (0.22 mmol) was added at room temperature to a stirred solution of 5 (85 mg, 0.22 mmol) and isopropyl alcohol (0.30 mmol) in dichloromethane (1.5 mL). After 1 h, pyridine-water (1:2, 1.5 mL) was added and the mixture was then diluted with dichloromethane. The solids were removed by filtration using a Celite filter bed. Removal of the solvents was achieved by azeotropic co-evaporation with several portions of toluene. The yield of crude glycoside (90%) was estimated from the ^1Hmr (CDCl_3) spectrum by comparing the relative intensities of the signals of the methyl groups in the aglycon with those for the O-acetyl signals. De-O-acetylation,

followed by treatment with Dowex 1 x 8 (OH^-), showed the product to be a 5:4 mixture of the α and β -D-glucopyranosides as determined from the relative intensities of their anomeric doublets (αH_1 : δ 5.01, J 3.5 Hz; βH_1 : δ 4.52, J 7.5 Hz. Under the same conditions, but with a reaction time of 15 h, a quantitative yield of a mixture of the isopropyl α - and β -glucopyranosides was recovered. The α/β ratio was 85:15.

Anomerization Reactions

Stannic chloride (1.0 mole equivalent) was added with vigorous mixing, at zero time, to a solution of the acylated isopropyl β -D-glucopyranoside (22, 24 and 25) (0.1 M) in pure dry chloroform (except in the case of the reaction of 22 catalyzed by an equimolar amount of acetic acid). The sample was transferred, via a syringe, to a thermostated ($25 \pm 0.2^\circ$) polarimeter cell and the rotation was followed until it reached a constant maximum. The reaction mixture was neutralized and processed as described above. The pseudo-first-order rate constants for the $\beta \rightarrow \alpha$ anomerization were determined using the standard integrated polarimetric rate expression and the half-times of reaction calculated from these constants.

The ^1Hmr spectra of the isolated reaction products were, in all cases, consistent with a mixture of isopropyl 3,4,6-tri-O-acetyl-2-O-acyl- α - and β -D-glucopyranosides

in an α/β ratio of near 85:15.

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